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THE UNIVERSITY OF ALBERTA

GENTAMICIN RESISTANCE IN PSEUDOMONAS AERUGINOSA

by

(C) RACHIT HARAPHONGSE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL BACTERIOLOGY

EDMONTON, ALBERTA

FALL, 1974

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Gentamicin Resistance in Pseudomonas aeruginosa", submitted by Rachit Haraphongse in partial fulfilment of the requirements for the degree of Master of Science.

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Supervisor

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Date May 22 1974

ABSTRACT

Fourteen clinical-isolates of Pseudomonas aeruginosa from the University Hospital, Edmonton were studied as representative gentamicin resistant strains. The MIC of gentamicin for these strains ranged from 6.25 ug/ml to 100 ug/ml. The strains were cross resistant to other aminoglycoside antibiotics (streptomycin, kanamycin, sisomicin, BB-K8 and tobramycin), their pyocine types were 1c, 3, 12, 16 and one was non-typable. Isolated ribosomes were sensitive to gentamicin and tobramycin in that amino acid incorporation in cell-free systems was prevented by these drugs. The gentamicin resistance could not be transferred to various recipients by conjugation, although extrachromosomal DNA was demonstrated from strains 8803 and 13934 by analytical CsCl gradient centrifugation. Inactivation of gentamicin by acetylation, adenylation or phosphorylation was not detected. Bioassay and paper chromatography of extracted gentamicin failed to demonstrate obvious inactivation. Whole cell uptake of radiolabelled gentamicin was examined after purification of the ^{3}H-gentamicin. A sodium azide insensitive phase of uptake was detected and ascribed to cell envelope binding of the gentamicin. Evidence is presented that sodium azide sensitive uptake corresponded with intracellular gentamicin accumulation and was necessary to cause cell death. A close relationship existed between azide-sensitive uptake and the MIC of a strain. A strain which was made more gentamicin resistant by growth in the presence of a low concentration of gentamicin, showed a corresponding decline in uptake of gentamicin. The possibility that uptake occurring at the MIC was secondary to some other inhibitory activity of gentamicin was shown to be unlikely in that a strain resistant to gentamicin by another mechanism accumulated

gentamicin below its MIC value. From these findings, it is concluded that the resistance to gentamicin in P. aeruginosa is most likely due to defective permeation of the drug.

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LIST OF ABBREVIATIONS

A	: absorbance
Acetyl Co A	: acetyl coenzyme A
AMP	: adenosine monophosphate
ATP	: adenosine triphosphate
cm	: centrimeter
cpm, CPM	: counts per minute
CTP	: cytidine triphosphate
CsCl	: cesium chloride
dAMP	: deoxyadenosine monophosphate
DEAE	: diethylaminoethyl
DNA	: deoxyribonucleic acid
dpm	: disintegrations per minute
EDTA	: disodium ethylene diamine tetraacetate
g	: gram
GC	: guanine plus cytosine content
GTP	: guanosine triphosphate
HCl	: hydrochloric acid
M	: molar
Mg	: magnesium
mM	: millimolar
mg	: milligram
ml	: milliliter
mCi/mmole	: millicuri per millimole
MIC	: minimal inhibitory concentration
OD	: optical density
nm	: nanometer
ng	: nanogram
RNA	: ribonucleic acid
nmole	: nanomole
NH_4Cl	: ammonium chloride
Str, Sm	: streptomycin
Str-R mutant	: streptomycin-resistant mutant

TCA	: trichloroacetic acid
TTP	: thymidine triphosphate
Tris	: Tris (hydroxymethyl) aminomethane
Sp ac	: specific activity
u/mg	: unit per milligram
uCi/umole	: microcuri per micromole
ug	: microgram
v/v	: volume per volume
w/v	: weight per volume

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INTRODUCTION AND LITERATURE REVIEW

Aminoglycoside antibiotics are a group of drugs that contain both amino sugar and aminocyclitol moieties except for spectinomycin, which contains an inositol with two of the hydroxyl groups substituted by methylamine moieties and kasugamycin which contains an inositol, a hexahydroxycyclohexane. The component rings of aminoglycoside antibiotics are linked via glycosidic linkage. There are two important subclasses; those containing streptidine, and those containing deoxystreptamine moieties. The streptidine antibiotics include streptomycin and its derivatives, hydroxystreptomycin, dihydrostreptomycin, mannosidostreptomycin and bleusomycin. Within the deoxystreptamine group there are two recognizable subclasses: in one, the substituents on the deoxystreptamine ring are attached in adjacent ("1, 2") positions (2 neomycins, 2 paromomycins, hybrimycins, lividomycins); and in the other the substituents are attached to nonadjacent hydroxyl groups ("1, 3"), (3 kanamycins, 4 gentamicins and tobramycin). (Rinehart 1969).

The action of the aminoglycoside antibiotics is inhibition of protein synthesis in susceptible cells. Tanaka et al. (1967), Masukawa and Tanaka (1968) provided a structural basis for miscoding of amino acid incorporation. Both deoxystreptamine and streptamine, although lacking antimicrobial activity, cause miscoding in cell-free extracts. Neither spectinomycin nor kasugamycin contains deoxystreptamine or streptamine and thus produces no miscoding (Umezawa et al. 1966, Tanaka et al. 1966, Tanaka et al. 1966). Kasugamycin inhibits protein synthesis by inhibition of phe-t RNA binding to ribosomes in response to poly U and thus is an inhibitor of the recognition process (Umezawa, H., 1966).

Its binding site is the 30S subunit (Sparling, 1970). Spectinomycin is bacteriostatic (Davies et al. 1965; Anderson, P. 1969). It inhibits protein synthesis through interaction with the 30S subunit (Anderson et al. 1967) and protein P_4 of that subunit (Bollen et al., 1969; Bollen and Herzog, 1970; Deiko and Tanaka, 1969). Its inhibition requires the presence of cytidylic or guanylic acid residues in templates. It interferes with mRNA: 30S subunit interactions occurring during translocation (Anderson et al., 1967).

Using cell-free extract from E. coli strains, Davies et al. 1964, and Van Knippenberg et al, 1965, showed that streptomycin and other aminoglycoside antibiotics can stimulate miscoding. Gentamicin induced the highest level of misreading of all the aminoglycosides tested (Davies et al., 1969). They vary widely in the degree of misreading produced as a function of their concentrations. Neomycin, kanamycin and gentamicin show a 3- to 5-fold increase in level of misreading as the drug concentration was raised from 10^{-6} to 10^{-4} M. This suggested that each of these drugs may act on more than one site. In contrast, little increase in misreading over the same concentration range was seen with streptomycin. This latter drug may act only on a single site (Davies and Davis, 1968). It has been shown that streptomycin acts by binding to the 30S ribosomal subunit and interfering with the function of the A site on the ribosome (Modolell, J. and Davis, B.D. 1969). By analogy, the other aminoglycoside antibiotics (kanamycin, neomycin, paromomycin, gentamicin and nebramycin) act in the same way, since their effects on cells and in cell-free systems that synthesize polypeptide share many common features (Davies, J., 1968). Using the procedures for the reconsitiution of ribosome subunits, Staehelin and Meselon (1966),

Traub et al, (1966), Traub and Nomura (1968) and Tanaka and Kaji (1968) have shown that the distinction between 30S ribosome from streptomycin-sensitive and streptomycin-resistant bacteria resides in the core proteins. Ozaki et al, (1969) identified a specific protein constituent of the 30S particle (P_{10}) controlled by the streptomycin-locus in E. coli. The results indicated that 30S ribosomal particles reconstituted with protein P_{10} from Sm-resistant strain and all other proteins from the Sm-sensitive strain were resistant to Sm. Conversely, particles reconstituted using P_{10} from Sm-sensitive and all other proteins from the Sm-resistant strain were sensitive to the antibiotic. Thus alteration in the P_{10} protein which accompanies mutation from sensitivity to resistance abolishes the binding ability of this structure for streptomycin. Amino acid analyses of protein S_{12} (P_{10}) from several str-R mutants have indicated that some have a specific lysine residue replaced by asparagine, threonine, or arginine, and others have an arginine-for-lysine replacement at a different site in the protein (Funatsu and Wittmann 1972).

Mutation was generally the first mechanism of resistance to antibiotics studied and results in alteration of a cellular component such that the antibiotic either does not reach or does not interact normally with its target site within the cell. These mutants were obtained as spontaneous or mutagen-induced strains which were selected by exposure to a high concentration (>100 ug/ml) of drug. The frequency of appearance of spontaneous streptomycin or kanamycin-resistant mutants in the laboratory is in the order of one in 10^9 - 10^{10} cells per generation (Davies, J. 1971). It is not surprising that antibiotic resistant mutants are relatively rare among clinical isolates. Tanaka

(1970) claimed to have found altered ribosome in 6 strains of clinical isolates of P. aeruginosa, whose MIC was higher than 100 ug/ml; however, these strains have been shown to contain an enzyme that inactivates gentamicin by acetylation (Brzezinska et al. 1972).

Clinical isolates of enterococci exhibit two types of resistance to streptomycin. Moderately high level resistance (MIC 62 - 500 ug/ml) which occurs in most natural isolates and can be considerably overcome by the addition of penicillin. The mechanism of this resistance appears to be due to a relative impermeability of the organism to streptomycin since it is reversed by agent which inhibit cell wall synthesis and uptake of labelled streptomycin is increased in the presence of penicillin (Moellering et al. 1970). Very high level resistance (MIC >2000 ug/ml) and lack of penicillin-streptomycin synergism appears to be due to ribosomally mediated streptomycin resistance. By dissociating the ribosomes from sensitive and resistant bacteria into 30S and 50S subunits and then reassociating the subunits in various combinations, it was found that the streptomycin-sensitive site is located in the 30S subunit. Mutation to streptomycin resistance changes this subunit in such a way that it is no longer susceptible to antibiotics (Zimmerman et al. 1971).

Some clinical isolates of P. aeruginosa that are streptomycin resistant (MIC 20,000 ug/ml) have been reported to have resistant ribosomes , containing no streptomycin-inactivating enzymes (Tseng et al. 1972).

An alteration in target site (the ribosome) is not the only mechanism by which resistance to aminoglycoside antibiotics can develop, and in natural isolates the most important mechanism of resistance is

inactivation of the antibiotic due to the presence of R-factors. An enzymatic basis for this resistance was first indicated by the study of Okamoto and Suzuki (1965), who found that extract of one strain of E. coli carrying an R-factor inactivated dihydrostreptomycin and chloramphenicol in the presence of ATP and acetyl coenzyme A respectively. Cell-free extract of another strain carrying an R-factor (E. coli K12 - R5) also inactivated kanamycin A in the presence of acetyl CoA. Subsequently, extensive work has been done on inactivating enzymes.

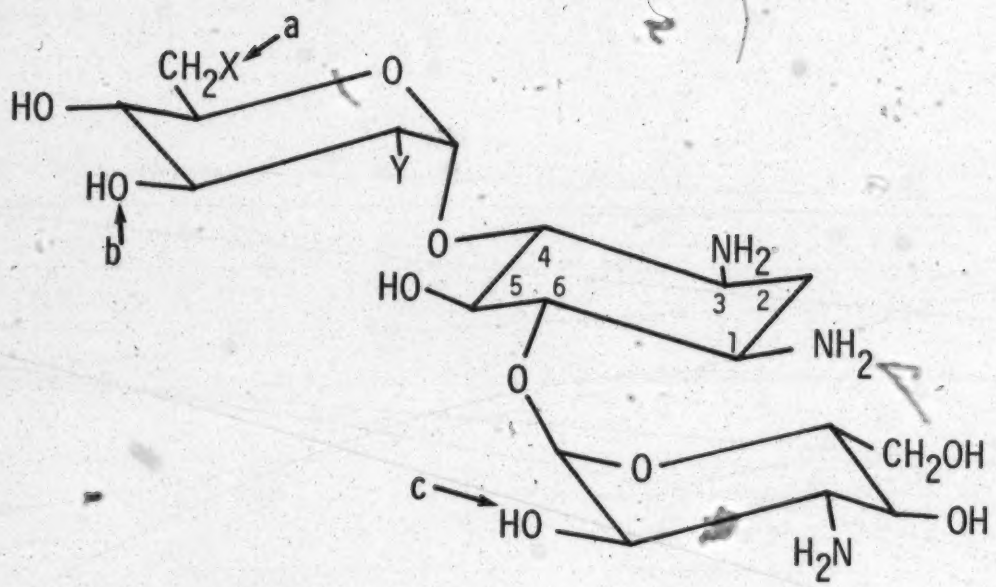
The enzymatic inactivation of aminoglycosides is monitored by measuring the reduced potency of the antibiotic in inhibiting the growth of sensitive strains of bacteria following incubation in the presence of a cell-free extract and coenzyme A or ATP. This microbiological assay can only measure the modification of a substance that is an antibiotic, and any potential modifications that do not result in complete inactivation of the antibiotic would not be detected. A cation-exchange paper (phosphocellulose) binding assay is a very sensitive method by which to detect the enzymatic inactivation of aminoglycosides (Davies, J. et al, 1971). By this method it is possible to detect as little as 10 ng of an aminoglycoside by assaying the antibiotic in the presence of the appropriate substrate at high specific activity. The aminoglycoside antibiotics are strongly basic compounds and are able to bind quantitatively to cation-exchange paper. By using ^{14}C ATP or α - ^{32}P ATP, $\gamma^{32}P$ ATP, or ^{14}C-acetyl coenzyme A, the transfer of a ^{14}C AMP, α ^{32}P AMP, ^{32}P or ^{14}C-acetate moiety to any aminoglycoside in the presence of a suitable cell-free extract can readily be detected. Presently, nine enzymes have been identified which inactivate the aminoglycoside antibiotics: three are acetylating

enzymes, four are phosphorylating enzymes, and two are adenylating enzymes (Benveniste and Davies 1973).

1. Kanamycin acetyltransferase: This enzyme, first reported by Okamoto and Suzuki (1965), inactivates kanamycin by acetylation, since a cell-free extract from an E. coli strain carrying R-factor R-5 inactivated kanamycin A in the presence of acetyl coenzyme A. Subsequently, Umezawa et al. (1967) purified the inactivated kanamycin and showed that the 6-amino group of its 6-amino-6-deoxy-D glucose moiety was acetylated (Figure 1). Okanishi et al. (1967), by using a microbiological assay found that the enzyme did not inactivate kanamycin B, kanamycin C, neomycin or paromomycin. Using the cation exchange paper binding assay in the presence of ^{14}C-acetyl coenzyme A with a partially purified acetylating enzyme, Benveniste and Davies (1971b) found that besides kanamycin A, kanamycin B, neomycin, gentamicin C_{1a}, gentamicin C_2, and sisomicin were also substrates for kanamycin acetyltransferase. Gentamicin A, gentamicin C_1, and paromomycin are also potent inhibitors of the enzyme, probably because they compete with the substrate for the active site on the enzyme.

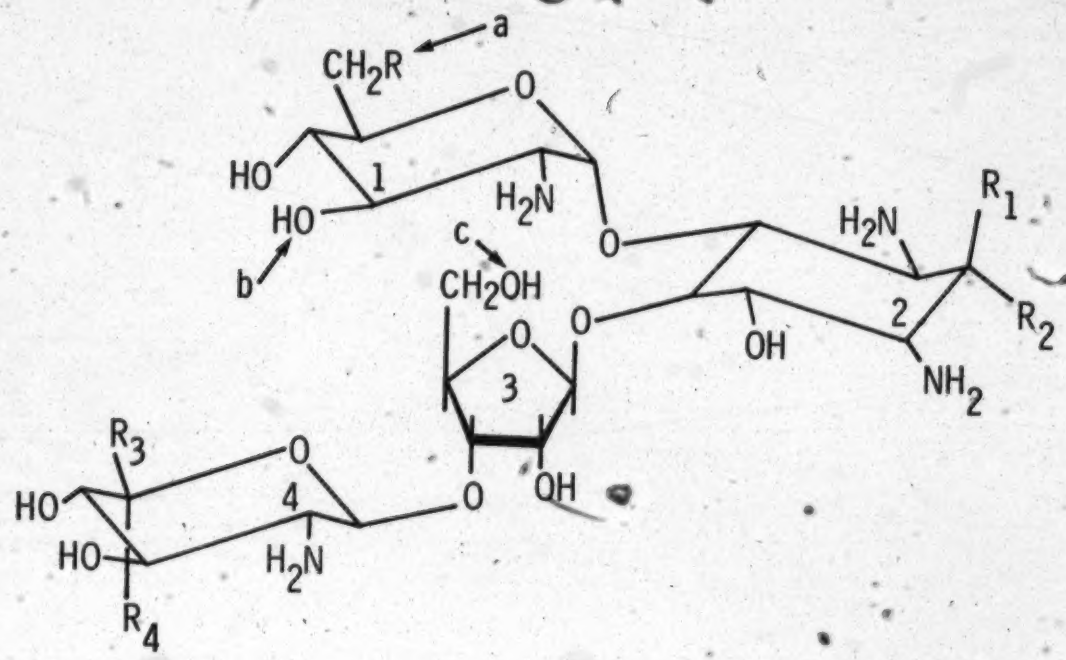
The site of acetylation of all these compounds has not been demonstrated. From the fact that neomycin B and C are acetylated, but paromomycin is not, and the only difference between these antibiotics is the presence or absence of an amino group at the 6 position of the hexose unit attached to deoxystreptamine, the acetylation of neomycin must occur on the amino group at the 6 position (Figure 2).

Although gentamicin C_{1a}, neomycin B, tobramycin, and kanamycin are all substrates for the acetylating enzyme, the R-factor containing strain is almost completely sensitive to these antibiotics. These



		X	Y
KANAMYCINS	A	NH_2	OH
	B	NH_2	NH_2
	C	OH	NH_2

FIGURE 1: The structure of kanamycins A, B and C. The arrows indicate the sites of N-acetylation by kanamycin acetyltransferase (a), the site of O-phosphorylation by neomycin-kanamycin phosphotransferase (b), and the site of O-adenylation by gentamicin adenyltransferase (c). (Benveniste and Davies 1973).



	R	R_1	R_2	R_3	R_4
NEOMYCIN B	NH_2	H	H	H	CH_2NH_2
NEOMYCIN C	NH_2	H	H	CH_2NH_2	H
HYBRIMYCIN A1	NH_2	OH	H	H	CH_2NH_2
HYBRIMYCIN A2	NH_2	OH	H	CH_2NH_2	H
HYBRIMYCIN B1	NH_2	H	OH	H	CH_2NH_2
HYBRIMYCIN B2	NH_2	H	OH	CH_2NH_2	H
PAROMOMYCIN	OH	H	H	CH_2NH_2	H
				H	CH_2NH_2

FIGURE 2: The structure of the neomycins, paromomycin. Neamine (or puromine) consists of rings 1 and 2, neobiosamine of rings 3 and 4. The arrows indicate the N-acetylation of kanamycin acetyltransferase (a), and O-phosphorylation by neomycin-kanamycin phosphotransferase (b) by lividomycin phosphotransferase. (Benveniste and Davies 1973).

purified N-acetylated antibiotics retain substantial antibiotic activity in that they inhibit the growth of a sensitive E. coli strain and inhibit R17 RNA-directed protein synthesis in vitro, although they are less potent (10- to 20-fold) than their unacetylated parent compounds. In contrast, N-acetyl kanamycin A is not able to inhibit the growth of either a sensitive E. coli strain or in vitro protein synthesis. Thus, kanamycin acetyltransferase does not inactivate all of the antibiotics that it modifies.

2. Gentamicin acetyltransferase 1.

This enzyme, first reported by Mitsuhashi et al, (1971), inactivates gentamicin by acetylation, since a cell-free extract from a Pseudomonas strain inactivated gentamicin in the presence of acetyl coenzyme A. A gentamicin-resistant, tobramycin-sensitive strain of P. aeruginosa (P. aeruginosa 130) produces an enzyme which, in the presence of ^{14}C-acetyl coenzyme A, transfers the labeled acetate moiety to antibiotics of the gentamicin C complex (Brzezinska et al,. 1972). This enzyme can be isolated from a cell-free extract of P. aeruginosa 130 by precipitation of nucleic acid, ammonium sulfate fractionation, and DEAE chromatography. It has a high specificity for gentamicin C_1, C_{1a}, C_2, sisomicin, and a very slight activity towards kanamycin B and tobramycin. Neomycin B, Kanamycin A and C are not inactivated by gentamicin acetyltransferase 1. None of these antibiotics are inhibitors of the acetylation of gentamicin C_{1a}.

The product of the acetylation of gentamicin C_{1a} by kanamycin acetyltransferase, 6-N-acetyl-gentamicin C_{1a}, can be acetylated by this enzyme; the resulting diacetate of gentamicin C_{1a} is inactive as an antibiotic. Physical and chemical studies of the product of acety-

lation of gentamicin C_{1a} have shown that the acetylation occurs on the 3 amino group of the 2-deoxystreptamine (Figure 3). The product, 3-N-acetyl gentamicin C_{1a}, is about 500 times less active than gentamicin C_{1a} in bacterial growth inhibition tests or in R17 RNA-directed polypeptide synthesis in vitro.

P. aeruginosa 130 has been shown to transfer resistance to three drugs (gentamicin, streptomycin and sulfonamide) by conjugation to 2 P. aeruginosa recipients (strain 280 and strain 1310) at high frequencies (Bryan et al., 1973) but not to an E. coli K12 recipient.

3. Gentamicin acetyltransferase II.

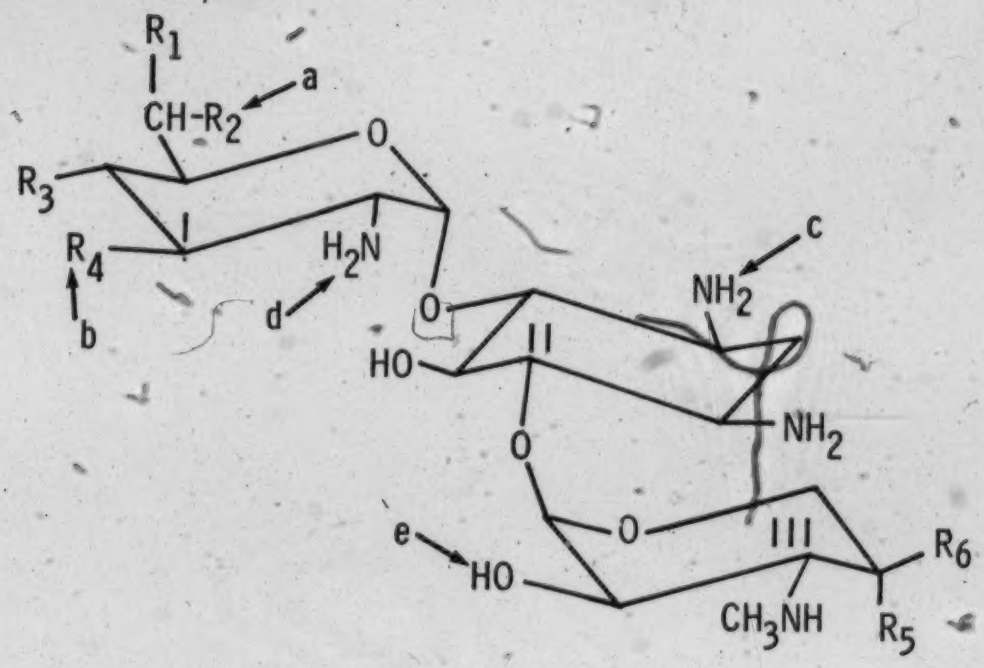
Cell-free extracts of antibiotic-resistant Providencia strains acetylate the gentamicin C complex and tobramycin but none of the other aminoglycoside antibiotics. The acetylation might occur on the 2'-amino group in the 3'-deoxyaminoglycosides (Figure 3) (Benveniste and Davies, 1973).

These three preceding aminoglycoside acetylating enzymes can be differentiated by their substrate specificities, pH optima and chromatographic techniques.

4. Streptomycin-spectinomycin adenyltransferase.

This enzyme was first reported by the studies of Okamoto and Suzuki (1965), who found that a cell-free extract of an E. coli strain carrying an R-factor inactivated streptomycin in the presence of magnesium and ATP. This work was confirmed and extended independently by Takasawa et al. (1968), Umezawa et al. (1968a), Yamada et al. (1968), and Harwood and Smith (1969).

Takasawa et al. (1968) and Umezawa et al. (1968a) reported that streptomycin could be inactivated by adenylation in the presence of



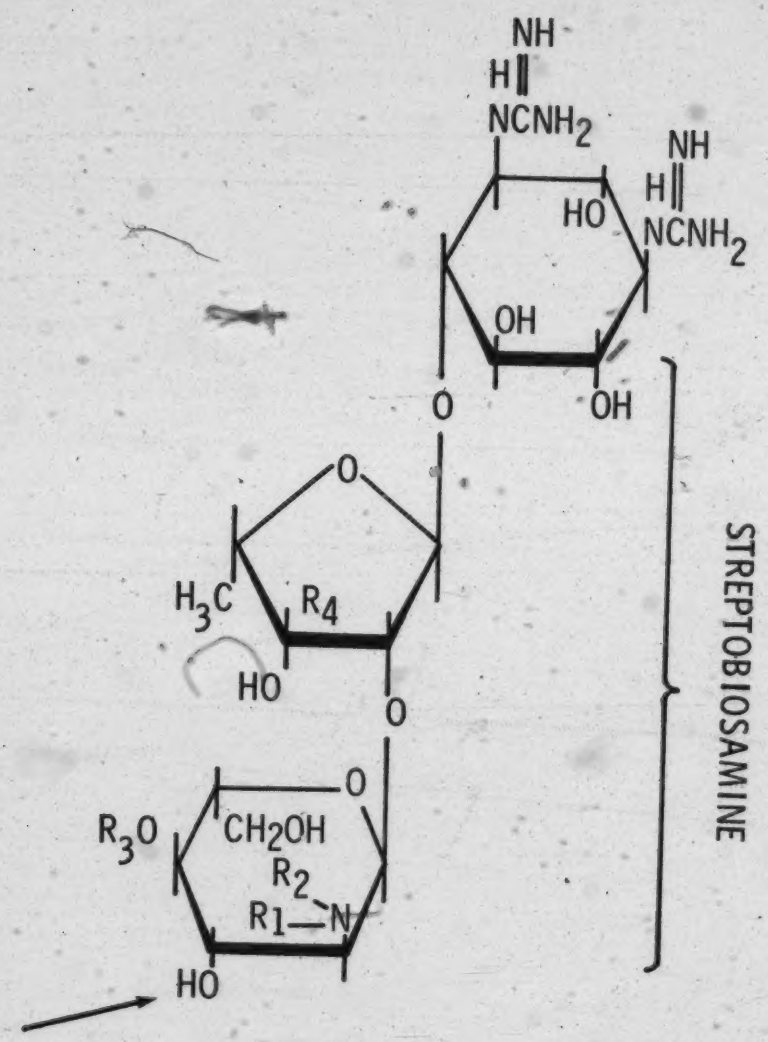
	R_1	R_2	R_3	R_4	R_5	R_6
GENTAMICINS A	H	OH	OH	OH	H	OH
C_{1a}	H	NH_2	H	H	OH	CH_3
C_2	CH_3	NH_2	H	H	OH	CH_3
C_1	CH_3	$NHCH_3$	H	H	OH	CH_3

FIGURE 3: The structure of gentamicins. Ring I is purpurosamine, II is 2-deoxystreptamine, and III is gentosamine (gentamicin A) or garosamine (gentamicin Cs). Sisomicin is 4,5-dehydrogentamicin C_{1a} (the purpurosamine ring is reduced). The arrows indicate where this group of antibiotics can be N-acetylated by kanamycin acetyltransferase (a), gentamicin acetyltransferase II (d), gentamicin acetyltransferase I (c), O-phosphorylated by neomycin-kanamycin phosphotransferase (b), and adenylylated by gentamicin adenyltransferase (e). (Benveniste and Davies 1973).

cell-free extract of E. coli ML 1629 carrying an R factor. Adenylation occurred not only in the presence of ATP but also ADP. The structure of the inactivated streptomycin was determined, and it was found that the hydroxyl group on C_3 of the N-methyl-L glucosamine moiety of streptomycin was adenylated (Figure 4).

Yamada et al. (1968) showed that in the presence of enzyme (a cell-free extract of the R factor containing strain, E. coli JE254), divalent cations (magnesium ion) and α -. ^{32}P ATP at pH 7.5 - 8.5, an adenyl (AMP) group was transferred to the 3'-hydroxyl group of the N-methyl-L-glucosamine moiety of streptomycin. GTP, CTP and TTP were not substrates for the enzyme. This enzyme did not inactivate kanamycin, neomycin, gentamicin or paromomycin. This enzyme, streptomycin adenyltransferase, was produced constitutively in the cell and was located within the periplasmic space of the bacteria since it could be released from cells subjected to osmotic shock. Streptomycin adenylate was no longer capable of binding to ribosomes isolated from streptomycin-sensitive strains of E. coli and was completely inactive as an antibiotic. Its antibacterial activity could be restored by incubation with venom phosphodiesterase.

Harwood and Smith (1969) showed that cell-free extract of E. coli B/RE130 eliminated the inhibitory effect of streptomycin, bleusomycin and spectinomycin but not of the other aminoglycosides. The requirements for this inactivation were determined to be: cell-free extract, ATP or dAMP, and magnesium ion. Chromatographic techniques with radioisotopes indicated that streptomycin, spectinomycin and bleusomycin were inactivated by adenylation resulting in the formation of an inactivated form of streptomycin, containing adenosine or deoxyadenosine,



	R_1	R_2	R_3	R_4
STREPTOMYCIN	CH_3	H	H	CHO
N-METHYLDIHYDROSTREPTOMYCIN	CH_3	CH_3	H	CH_2OH
N-DEMETHYLDIHYDROSTREPTOMYCIN	H	H	H	CH_2OH
MANNOSIDO - STREPTOMYCIN	CH_3	H	MANNOSE	CHO

FIGURE 4: The structure of streptomycin and its derivatives. The arrow indicates the site of enzymatic adenylylation or phosphorylation in this antibiotics. (Benveniste and Davies 1973).

phosphate, and streptomycin in equimolar amounts. The adenylate moiety was coupled to the streptobiosamine residue of the streptomycin molecule.

R factor-carrying strains of E. coli that inactivated streptomycin by adenylation were also resistant to spectinomycin. Benveniste et al. (1970), and Smith et al. (1970) reported that like streptomycin, spectinomycin was also inactivated by adenylation. The heat inactivation of a partially purified enzyme showed that both streptomycin and spectinomycin adenylating activities were destroyed at the same rate, and both activities were present in a partially purified protein fraction. Moreover, mutants obtained from this strain that were sensitive to either spectinomycin or streptomycin were shown to lack both enzymatic activities when tested in vitro, and revertants of these mutants selected for recovery of either streptomycin or spectinomycin resistance regained both activities. Therefore, the activation of the two drugs is catalyzed by the same enzyme. Actinamine, which is not an antibiotic but contains the D-threo-methyl-amino alcohol moiety, is a substrate for streptomycin-spectinomycin adenyltransferase. So it is possible that the adenylating enzyme transfers AMP to the D-threo-methyl-amino-alcohol moiety that is present in both streptomycin and spectinomycin (Figure 4 and Figure 5). Several derivatives of streptomycin such as dihydrostreptomycin, bleusomycin, and hydroxystreptomycin are also substrates for the enzyme.

5. Gentamicin adenyltransferase.

Martin et al. (1971) reported the isolation of the first gentamicin-resistant strains of Klebsiella pneumoniae type 22. These strains were resistant to streptomycin, kanamycin, neomycin, ampicillin, tetra-

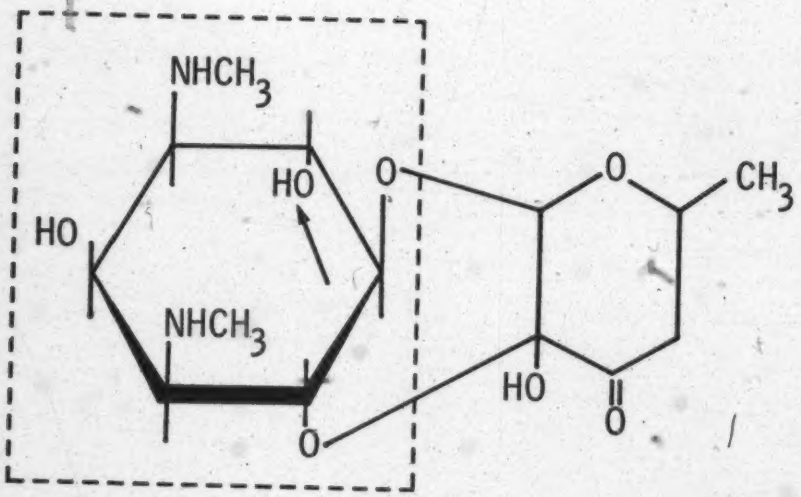

NHCH3
HO
HO
O
O
CH3
NHCH3
HO
O
O

FIGURE 5: The structure of spectinomycin. The arrow indicates the site of adenylation; actinamine, the residue within the dotted box, is a substrate for streptomycin-spectinomycin adenyltransferase. (Benveniste and Davies 1973).

cycline and chloramphenicol. One of these strains (K. pneumoniae #3038) was shown by Benveniste and Davies (1971a) to contain an R factor which mediated resistance to the antibiotics of the gentamicin C complex. Simultaneous transfer of resistance to neomycin, kanamycin, streptomycin, chloramphenicol, tetracycline, ampicillin and sulfonamide occurred. Osmotic shockate of this strain inactivated gentamicin C_{1a} in the presence of ATP and magnesium ion. It was shown by chemical assay that only ^{14}C ATP or α - ^{32}P ATP could transfer their radioactively labeled moieties to gentamicin C_1, C_{1a}, C_2, A and kanamycin in the presence of osmotic shockate. Inactivated gentamicin C_{1a} did not inhibit in vitro protein synthesis and did not inhibit the growth of sensitive strains of E. coli. Incubation of inactivated gentamicin with snake venom phosphodiesterase yielded the active parent compound, whereas alkaline phosphatase had no effect. Therefore these antibiotics are inactivated by adenylation. This enzyme did not inactivate streptomycin or spectinomycin. This enzyme was found to inactivate 3', 4'-dideoxykanamycin B in the presence of ATP (Naganawa et al. 1971). The structure of the inactivated antibiotic is shown to be 3', 4'-dideoxykanamycin B - 2" adenylate by nuclear magnetic resonance study. The gentamicins are presumable adenylated on the same position (Figure 3).

6. Streptomycin phosphotransferase.

7. Neomycin-kanamycin phosphotransferase 1.

8. Neomycin-kanamycin phosphotransferase 11.

Umezawa et al. (1967), Okanishi et al. (1968), and Kondo et al. (1968) reported that the 3-hydroxyl group of 6-amino-6 deoxy-D-glucose moiety of kanamycin and the 3-hydroxyl group of glucosamine moiety of

paromamine were phosphorylated by cell-free extract of E. coli K12-ML 1629 (Figures 1 and 2). These inactivated products and dihydrostreptomycin inactivated by the same cell-free extract were converted to the original antibiotics by treatment with alkaline phosphatase.

Umezawa et al. (1968b), Doi et al. (1968a) reported that supernatant fluids from centrifugation of cell-free extracts at 105,000 x g contained enzymes inactivating kanamycin, neomycin and streptomycin in the presence of ATP. The inactivated kanamycin was shown to be kanamycin-3'-phosphate in which the C_3 hydroxyl group of 6-amino-6 deoxy-D glucose was phosphorylated. Similarly, the cell-free system of clinical isolates of drug-resistant Staphylococcus aureus inactivated kanamycin, and the inactivated product was identified with kanamycin-3'-phosphate (Doi et al. 1968b).

Ozanne et al. (1969) reported that streptomycin-resistant and spectinomycin-sensitive E. coli JR35, carrying an R factor produced two enzymes, one which phosphorylated streptomycin and another which phosphorylated the antibiotics of the neomycin-kanamycin group. Streptomycin phosphorylation requires only streptomycin and magnesium-ATP; the divalent cation requirement can also be satisfied with zinc or manganese, unlike the kanamycin phosphorylation which has an absolute requirement for magnesium. Streptomycin phosphotransferase will phosphorylate methylstreptobiosamine, N-methyl dihydrostreptomycin, but it will not use any of the other aminoglycoside antibiotics or spectinomycin as substrates. The site of enzymatic phosphorylation of streptomycin was at the 3' hydroxyl group of the N-methyl-L glucosamine ring (Figure 4). Streptomycin phosphate is inactivate as an antibiotic when tested against streptomycin-sensitive or streptomycin-dependent

strains of E. coli, or when tested for its effect on polypeptide synthesis in vitro (Yamada et al. 1968).

Neomycin-kanamycin phosphotransferase, mentioned above, is able to inactivate vistamycin, kanamycin A, B, C and gentamicin A, all of which contain the 3'hydroxyl group. Those related aminoglocysides that do not contain this hydroxyl group, such as gentamicin C_{1a}, C_1, C_2, sisomicin, tobramycin, lividomycin B, and the synthetic antibiotics 3', 4' dideoxykanamycin, 3', 4'-dideoxyvistamycin, 3'-deoxykanamycin B, and 3', 4'-dideoxyneamine, are not phosphorylated by this enzyme and are potent inhibitors of the phosphorylation reaction (Davies et al. 1971). This enzyme can be divided into two separate enzymes, neomycin-kanamycin phosphotransferase I and neomycin-kanamycin phosphotransferase II. Neomycin-kanamycin phosphotransferase I will phosphorylate the substrates mentioned above, whereas neomycin-kanamycin phosphotransferase II will also phosphorylate butirosin. These enzymes also differ in their response to inhibitors of phosphorylation. For example, tobramycin inhibits the phosphorylation of neomycin catalyzed by neomycin-kanamycin phosphotransferase I, but has no effect on the reaction catalyzed by neomycin-kanamycin phosphotrasferase II (Benveniste and Davies 1973).

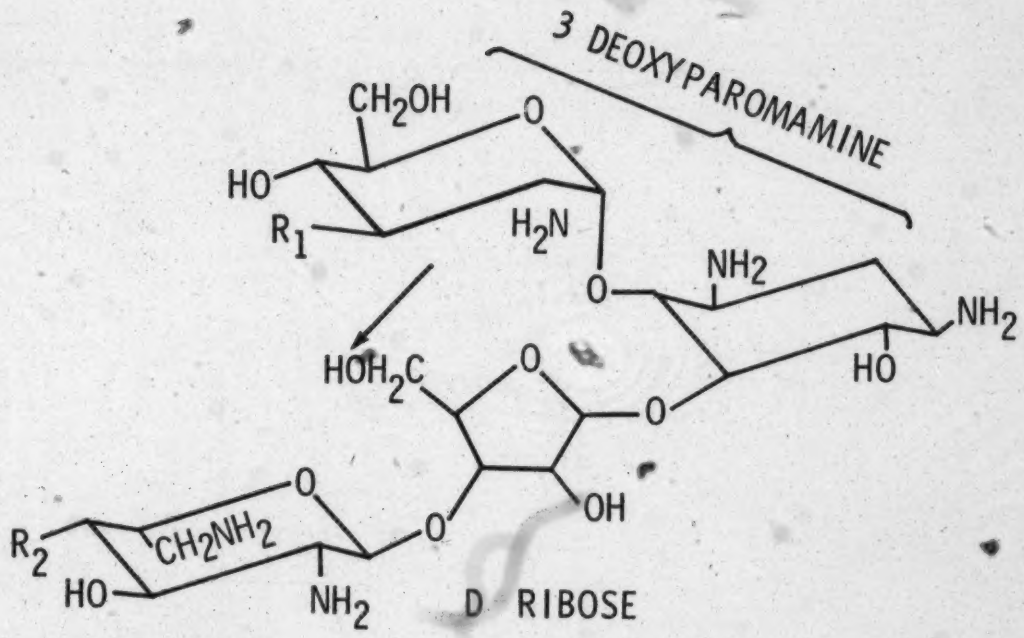
9. Lividomycin phosphotransferase.

Lividomycin A, B, new aminoglycoside pentasaccharide antibiotics containing 2 deoxystreptamine, are nbt inactivated by neomycin-kanamycin phosphotransferase because they lack a 3'hydroxyl group (Davies et al. 1971). By use of a cell-free extract from P. aeruginosa TI-13, a highly lividomycin-resistant strain of clinical origin, the inactivation of lividomycin was found to be caused by the formation of

a monophosphorylated product of the drug (Kobayashi et al. 1972). Yamaguchi et al. (1972a, b) have reported the presence of an enzyme in a lividomycin-resistant strain of E. coli carrying an R-factor which phosphorylates the lividomycin on the 5"-hydroxyl group of the D-ribose moiety (Figure 6). This enzyme can be purified by fractionation with ammonium sulfate and Sephadex G-100 column chromatography. It phosphorylates neomycin, paromomycin and vistamycin, but does not inactivate the kanamycins, streptomycin, or the gentamicin C complex.

Resistance to aminoglycoside antibiotics in clinical isolates of gram-negative bacteria is due mainly to the presence of enzymes that specifically modify the antibiotics so they can no longer interact with their target in the cells. Synthesis of these enzymes is determined by R-factors at least in most instances. R-factors (drug resistance factors) consist of autonomously replicating, non-homogenous, extrachromosomal units of DNA that are both distinct genetically and physically (Falkow et al. 1966, Anderson, 1968). An R-factor is an independent linkage group, composed of genes determining drug resistance (r determinants) associated with an RTF (resistance factor or transfer factor) conferring the ability to conjugate and to transfer the factor to a new host (Watanabe and Fukasawa 1961).

Perry (1969) showed that in group H Streptococci only the streptomycin-sensitive Challis strain accumulated significant amounts of ^{14}C-streptomycin, whereas the streptomycin-resistant mutants accumulated very little streptomycin. This evidence suggested that alteration in permeability could be one of the mechanisms involved in streptomycin resistance. Some clinical isolates of P. aeruginosa that are streptomycin resistant have been reported to contain no streptomycin-



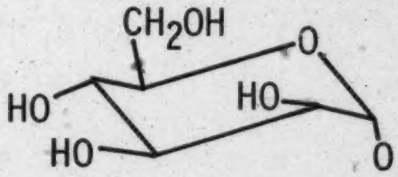
	R_1	R_2
LIVIDOMYCIN A	H	
B	H	OH

FIGURE 6: The structure of lividomycin A, B. The arrow indicates the site of phosphorylation. (Yamaguchi et al 1972b, Benveniste and Davies 1973).

inactivating enzymes. These strains have also been shown to contain ribosomes susceptible to streptomycin and to be free of R-factors. The mechanism of resistance appears due to reduced permeability to the drug (Tseng et al. 1972).

P. aeruginosa are motile bacilli with polar flagella. It is ubiquitous being found widely distributed in nature, in soil, water, sewage and air. They can be isolated from feces of symptomless human carriers and are resistant to soap and water and to many disinfectants (Jellard and Churcher 1967). The organism can be found in the hospital environment, equipment and on the hands of personnel (Shulman et al. 1971). Therefore, it is quite impossible for any patient to escape exposure to the organism. Premature babies who are deficient in serum bactericidal activity to gram-negative bacilli (Gitlen et al. 1963), immunosuppressed patients, patient with burns, cystic fibrosis or any chronic pulmonary disease, or cancer, are susceptible to Pseudomonas infection. P. aeruginosa produces an impressive array of toxins such as a hemolysin which is toxic to human cells, lipase (Liu et al. 1961), and enterotoxin (Kubota and Liu 1971). It also elaborates protease and lecithinase (Liu et al. 1961, Liu 1966) which may be responsible for the characteristic skin lesions (Yow and Townsend 1953, Mull and Callahan 1965, Liu 1966a, Meinke and Berke 1970). Furthermore, a partially purified protease, an elastase, has been shown to produce pulmonary hemorrhage in mouse experiments (Meinke et al., 1970). Endotoxin (the lipopolysaccharide layer) has a minor role in the pathogenesis of P. aeruginosa infection in mice (Liu et al. 1961) However, in human bacteremic infection due to P. aeruginosa, circulating endotoxin has been identified and its presence in the blood has been

correlated with increased mortality (Jones 1968). Slime is capable of producing toxic manifestations, sustained leukopenia, and death of mice (Sensakovic and Bartell 1974). Another toxin, designated "lethal toxin or exotoxin" is found (Liu 1966b, Pavlovskis and Gordon 1972) and is capable of producing shock (Atik et al. 1968). The organism also produces hydrogen cyanide which is detectable in burn eschar or viscera of patients with overwhelming Pseudomonas infection (Goldfarb and Margard 1967). The above reasons explain why P. aeruginosa can produce severe infection.

P. aeruginosa is resistant to most available antibiotics. At present, gentamicin is one of the most useful antibiotics against P. aeruginosa. Gentamicin, a broad spectrum antibiotic complex, has been isolated from submerged fermentations of Micromonospora purpurea and Micromonospora echinospora (Weinstein et al. 1964). The complex consists of 3 components, designated C_1, C_2 and C_{1a} (Wagman et al. 1968). There are no detectable differences in the biological activities of the three gentamicin components (Weinstein et al. 1964). They are pseudotrisaccharides containing a garosamine and purpurosamine linked by glycoside bonds to 2-deoxystreptamine. The positions of linkage of garosamine and purpurosamine to the 2-deoxystreptamine ring are C-6 and C-4 respectively (Copper et al. 1971). It shows excellent in-vitro and in-vivo effectiveness against Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, Serratia marcescens, Proteus species, and P. aeruginosa (Bulgar et al. 1963). The first medical application of gentamicin was in March, 1962, at the University of Illinois College of Medicine to a patient with extensive burn and septicemia due to P. aeruginosa (Jackson 1969). Later reports illus-

trated the emergence of gentamicin-resistant P. aeruginosa during topical therapy (Snelling et al. 1970, Shulman et al. 1971).

One mechanism of gentamicin resistance in clinically isolated P. aeruginosa has been shown to be enzymatic acetylation (Mitsuhashi et al. 1971), Brzezinska et al. 1972). The strain, reported by Brzezinska, was found to contain an R factor which mediated resistance to gentamicin, streptomycin and sulfonamide (Bryan et al. 1973).

The percentage of gentamicin-resistant P. aeruginosa is increasing at the University of Alberta Hospital. In a four-month period in 1973 11% of all strains were resistant to gentamicin and 31% moderately resistant (Bryan, unpublished data) using standard testing criteria (Bauer et al. 1966). R factors were detected in 3.3% of 233 hospital isolates of P. aeruginosa using P. aeruginosa recipients in conjugation. Transferred markers included streptomycin, tetracycline, and sulfonamide resistance. No transferable resistance to gentamicin could be detected at the University of Alberta Hospital (Bryan et al. 1973). Therefore, it is quite interesting to study the mechanism of gentamicin resistance in clinically isolated P. aeruginosa in the University of Alberta Hospital strains as they may not contain R-factors.

A good knowledge of the mechanisms of antibiotic inactivation or resistance in general and some knowledge of the structure-activity relationships among antibiotics may allow chemical design of new antimicrobial agents active against resistant bacteria. For example, synthesized 3'-deoxykanamycin (Umezawa, S. et al. 1971a) 3', 4' dideoxykanamycin B (Umezawa, H. et al. 1971b) and 3', 4' dideoxyneamine (Umezawa, S. et al. 1971b) were found to be active against resistant bacteria which produced neomycin-kanamycin phosphotransferase. Simi-

larly 5"-deoxylividomycin A and 5"-amino 5"-deoxylividomycin A were found to be active against resistant bacteria which produced lividomycin phosphotransferase (Yamamoto et al. 1972).

The antibiotic inactivations can also be eliminated by the use of the antibiotics in combination with inhibitors of the inactivation reactions. For example, the N-acetylation of kanamycin and related antibiotics is inhibited by the presence of paromomycin and gentamicin A (Benveniste and Davies 1971).

When resistance is due to a permeability defect, it may be overcome by modification of permeation in the bacteria by a second antibiotic that can alter the barrier of permeability. Also chemical design of antibiotics to take advantage of higher affinity transport mechanisms is another possibility.

Therefore, this study could have both beneficial academic and practical implications.

MATERIALS AND METHODS

1. Reagents: All chemicals were of reagent grade and were obtained from commercial suppliers. Ribonuclease A (6100 u/mg) and deoxyribonuclease (2000 u/mg) were obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Cesium chloride was obtained from Fisher Scientific Company. Lysozyme (18,000 u/mg, 1 unit produces a Δ OD_{450} of 0.001 min. at pH 6.24 at 25C. in 2.6 ml suspension of M. lysodeikticus) and sodium dodecyl sulfate were obtained from Sigma Chemical Company, St. Louis, U.S.A. Brij-58 (polyoxyethylene (20) cetyl ether) was obtained from Atlas Chemical Industries, Wilmington, Delaware, U.S.A. ^{14}C valine (50 mCi/m mole) was obtained from Schwarz/Mann. ^{14}C phenylalanine (54 mCi/m mole), acetyl-1 ^{14}C Coenzyme A (4 uCi/u mole), adenosine 5'-triphosphate, Tetra (Triethyl ammonium) salt [γ- ^{32}P] (6.93 uCi/u mole), and adenosine - ^{14}C (U) 5' - .3 (PO_4), 4 Na salt (4 uCi/u mole) were obtained from New England Nuclear, Boston, Massachusetts, U.S.A. Polyuridylic acid was obtained from Miles Laboratories, Inc., Illinois, U.S.A. R17 RNA from Dr. Jui-Teng Tseng, Department of Medical Bacteriology, University of Alberta, and E. coli K12 transfer RNA from Schwarz/Mann.

2. Antibiotics: Sensitivity discs were obtained from Baltimore Biological Laboratories. Gentamicin sulphate injectable (40 mg base/ml) was obtained from Schering Corporation Limited; tobramycin (1,000 ug Base/ml) from Lilly; Sisomicin (610 ug/mg) from Schering Corporation; BB-K8 from Bristol Laboratories (880 ug/mg). Gentamicin was tritiated by catalytic tritium exchange by the Radiochemical Centre, Amersham.

3. Buffers used were:

A. Buffer A: 10 mM Tris-HCl pH 7.8, 60 mM NH_4Cl, 10 mM Mg acetate, 6 mM 2-mercaptoethanol.

B. Buffer B: 50 mM Tris-HCl pH 7.8, 60 mM NH_4Cl, 7.5 mM Mg acetate, 6 mM 2-mercaptoethanol.

C. Standard buffer: 0.01 M Tris-HCl pH 7.8, 0.014 M Mg acetate, 0.06 M KCl, and 0.006 M 2-mercaptoethanol.

D. Mix 1: 10.0 ml of 2 M Tris-HCl pH 7.8, 2.0 ml of 1.4 M Mg acetate, 5.0 ml of 2 M KCl, 3.0 ml of 6.6×10^{-2} M ATP, Na salt, and 2.0 ml of 10^{-3} M GTP, Na salt. The mixture was stored in 1 ml aliquots at -20C.

4. The following media were used and obtained from the indicated sources:

Mueller-Hinton agar (BBL);

Trypticase soy (TS) broth and agar (TSA) (BBL);

1% Triphenyl tetrazolium chloride in nutrient agar No. 1 (Oxoid No. 1); medium for acetamide hydrolysis.

Base -	Peptone, bacto (Difco)	1	g
	Sodium chloride	5	g
	Monopotassium phosphate	2	g
	Phenol red	0.012	g
	Distilled water	1000	ml

The basal medium and 1% acetamide were adjusted to pH 6.8, tubed and sterilized at 121 C for 20 minutes. A 20% (w/v) solution of urea was sterilized by passage through a Seitz filter, and was added to tubes of basal medium and acetamide, after they were cooled to approximately 50 C, to produce a final concentration of 2% urea in the medium.

5. Organisms:

i. P. aeruginosa 130 (obtained from Dr. J. Davies, University of Wisconsin) was used as the positive control for the mating system and gentamicin acetyltransferase assay.

ii. P. aeruginosa 931 was used as the positive control for the streptomycin phosphotransferase assay.

iii. P. aeruginosa POW 151 (obtained from Dr. S. Kabins, Michael Reese Medical Center, Chicago, Ill.) was used as the positive control for the gentamicin adenylyltransferase assay.

iv. Recipient P. aeruginosa (280, 1310, 3503) and E. coli 1310 were rifampicin-resistant and gentamicin-sensitive strains.

v. The gentamicin-resistant strains of P. aeruginosa used in this study were isolated at the University of Alberta Hospital, Edmonton, Alberta in 1972.

6. Strain characterization:

P. aeruginosa strains were characterized by using the methods of Cowan and Steel (1965) except for acetamide hydrolysis (Buhlman et al, 1961) and growth in triphenyl tetrazolium chloride (Wahba and Darrell, 1965). DNA base ratio determination was carried out by the method of Owen et al. (1969). Pyocine typing was done by the Provincial Laboratory of Public Health using the method of Govan and Gillies (1969).

7. Disc sensitivity tests:

These were done by the procedure of Bauer et al. (1966) on Muller Hinton agar.

8. Minimal inhibitory concentration determinations:

In vitro testing of antibiotic susceptibility was done in TS broth using a conventional tube-dilution procedure. The volume of medium was

2.5 ml (in 18 x 125 mm tubes) and the inoculum was 0.1 ml of a broth adjusted to 0.05 A_{550} absorbance units (approximately 10^7 organisms). The end point was determined visually after about 18 hours of incubation at 37C.

9. Mating systems:

Recipient and donor strains were grown in TS broth and adjusted to an absorbance of 0.5 A_{550} unit/ml (Spectronic 20, Bausch & Lomb) prior to mixing. Five ml mating mixtures containing 2.5 ml of recipient and 2.5 ml of 1:10 dilution of the donor strain were added to a 150 ml medicine bottle which was then incubated flat side down in a 37C incubator. Mating times were 3 hour and overnight (18 - 20 hr). At the end of the mating period, several 0.1 ml volumes of 10-fold dilutions of the mating mixture were spread on TSA-containing rifampicin 100 ug/ml and gentamicin 1 ug/ml. The mating was also done by incubating the mixture at 37C for 3 hrs followed by vigorous overnight shaking. Samples were plated as given above.

10. Analytical CsCl gradient centrifugation of DNA:

This experiment was carried out according to Bryan et al. (1973). Cells were grown aerobically at 37C in 150 ml of TS broth with vigorous shaking until 0.5 A_{600} and then centrifuged at 5860 g for 20 minutes with the GSA rotor in a Sorvall RC 2-B centrifuge at 4C. The bacterial pellet was washed twice in Tris-EDTA-NaCl buffer (0.05 M Tris-HCl pH 8.0, 0.005 M EDTA and 0.05 M NaCl) and resuspended in 10.0 ml of 25% (w/v) sucrose in 0.05 M Tris HCl pH 8.0. One ml of freshly prepared lysozyme (10 mg/ml) was added to the bacterial suspension which was then incubated in an ice-water bath for 5 minutes. Two ml of EDTA (0.5 M, pH 8.2) was added to the suspension and the solution was

incubated an additional 5 minutes in the ice-water bath. The resulting spheroplasts were treated with Brij-58 (final concentration 0.5%w/v) and the solution incubated with ribonuclease (final concentration 50 ug/ml) at 37C for 60 minutes. At the end of incubation, 1 ml of 3% (w/v) sodium dodecyl sulfate (S.D.S.) was added to produce a final concentration of 0.2% (w/v) S.D.S. Five ml of chloroform and 10 ml of phenol, re-distilled and equilibrated with 2 x SSC [standard saline citrate (SSC) is 0.15 M NaCl, 0.015 M sodium citrate], were added to the solution, mixed thoroughly and centrifuged at 3021 g for 20 minutes with the JA-20 rotor in a Beckman J-21 centrifuge. The uppermost layer was collected and extracted as above until a clear supernate was obtained. The extracted DNA was dialyzed for 3 days against 2 x SSC containing 0.01 M EDTA, pH 8.0. The concentration of DNA was estimated spectrophotometrically (Unicam SP 1800), assuming an absorbance of 20 at 260 nm was equivalent to a concentration of 1 mg/ml DNA. Saturated CsCl was added to the DNA solution to give a final buoyant density of 1.71 ± 0.02 g/ml and the final DNA concentration was 5 ug/ml. Centrifugation was performed in a Beckman Model E analytical ultracentrifuge at 44,000 rpm with An - D Rotor for 25 - 30 hours at 25 C by Dr. J. Kinnear, Department of Microbiology, University of Alberta. During centrifugation ultraviolet photographs were taken and traced with a densitometer.

11. In vitro protein synthesis using cell free extracts.

Cells were grown aerobically at 37C in TS broth with vigorous shaking, harvested in logarithmic growth phase and washed in buffer A. Ribosomes and enzyme fractions were prepared by methods similar to those reported by Tseng et al. (1972). The bacteria was suspended in 6

ml of buffer A and subjected to ultrasonic vibration (Biosonik 111, Bronwill Scientific, Inc. 3/8" diameter probe) at 4C for 1 min. Deoxyribonuclease was added to the sonicated fluid to a concentration of 3 ug/ml. It was then centrifuged at 27,191 g for 30 minutes with the JA-20 rotor in a Beckman J-21 centrifuge at 4C. The supernate was dialyzed overnight at 4 C against buffer B (R17 RNA system) or standard buffer (poly U system). This fraction (S-30) was centrifuged at 208,000 g for 90 minutes with the SW 56 Ti rotor in a Beckman L2-65B ultracentrifuge at 4 C. The supernate is termed the S-100 fraction. The ribosomal pellet was resuspended in buffer B (R17 RNA system) or standard buffer (poly U system) to give a final concentration of 12 mg of ribosomes per milliliter.

R17 RNA system: R17 RNA-directed polypeptide synthesis was carried out according to Tseng et al. (1972). The reaction mixture contained: 50 mM Tris-HCl pH 7.8, 60 mM NH_4Cl, 7.5 mM magnesium acetate, 10 mM reduced glutathione, 1 mM adenosine triphosphate, 0.03 mM guanosine triphosphate, 5 mM creatine phosphate, 50 ug of creatine kinase/ml, 0.05 mM each of the 19 ^{12}C amino acids, 0.02 uM ^{14}C valine (50mCi/m mole), 0.7 mg of R17 RNA/ml, 1.0 mg of E. coli K12 transfer RNA (t RNA)/ml, 0.2 volume of S-100 fraction, and 0.2 volume of ribosome suspension. Gentamicin, if present, was used at 10 ug/ml.

Reaction mixtures were incubated at 34C for 30 min, and 50 ul was spread on an appropriate square of silica gel loaded paper (1.4 by 1.4 cm, Whatman SG81). A 50 ul amount of 1 N NaOH was added immediately to the wet paper to stop the reaction and to hydrolyze the ^{14}C valine-tRNA. When the papers appeared nearly dry, they were washed three times by dropping them into 100 ml of ice-cold trichloroacetic acid

solution (10% w/v), and agitating gently for 15 min each time by placing the ice bucket containing the flask with TCA on a rotary shaker. The papers were next sequentially washed in 50 ml each of ethanol-ether mixture (1:1, v/v) and ether. The flask was inverted over two or three paper towels. Papers were dried at room temperature, dropped into counting vials containing 10 ml of toluene scintillation fluid (4.0 g PPO, 0.1 g POPOP per liter of toluene) and counted using a Beckman LS-250 liquid scintillation counter.

Poly U system: Polyuridylic-acid directed polypeptide synthesis was carried out by a method similar to that reported by Nirenberg (1964). The reaction mixture contained: 2 M Tris-HCl pH 7.8, 1.4 M Mg Acetate, 2 M KCl, 6.6 x 10^{-2} M ATP, 10^{-3} M GTP, 5 mM creatine phosphate, 50 ug creatine kinase/ml, 0.2 mM each of the other 19 C^{12} amino acids, 0.8 mg of E. coli K 12 transfer RNA/ml, 0.2 volume of S-100 fraction, and 0.2 volume of ribosome suspension. The mixtures were pre-incubated at 37C for 30 min. Gentamicin was added to a final concentration of 1 ug/ml and tobramycin, if present, at 1 ug/ml and the mixtures were incubated for five more minutes. Twenty ug of poly U and 0.0925 u mole ^{14}C phenylalanine were added to the mixtures. A 50 ul sample was taken from the mixture that contained no antibiotic as the zero time sample. All the mixtures were then incubated at 34C for 30 minutes. Samples were processed as described for R17-RNA system.

I2. Examination for enzymatic inactivation of gentamicin by cell-free extracts.

Cell extracts (S-30 fraction) used in this experiment were prepared by the method described in the amino acid incorporation study.

A. Microbiological assay. The reaction mixture consisted of the

following: 0.8 ml of S-30 fraction, 40 ug of gentamicin and 400 ug of acetyl coenzyme A (for gentamicin acetylating activity study) or 400 ug of ATP (for gentamicin adenylating and phosphorylating activity study) in a total volume of 1 ml. After incubation at 37C for 6 hrs, the mixture was heated in an 80C water bath for 15 minutes to kill the contaminating vegetative bacteria. The mixture was then examined for antimicrobial activity by a broth dilution technique using P. aeruginosa strain 280 (MIC = 0.25 ug/ml), as the test organism.

B. Chemical assay. Assay for acetylating and adenylating activity was done according to Benveniste and Davies (1971b) and Ozanne et al (1969) respectively except that S-30 fraction (see methods for polypeptide synthesis) was used in place of osmotic shockate. The reaction mixture contained 20 ul of S-30 fraction, 5 nmoles of [^{14}C] acetyl CoA (specific activity = 4 uCi/u mole) for the acetylating assay or 5 nmoles of ^{14}C ATP (sp. ac. = 4 uCi/u mole) for the adenylating assay; 5 nmoles of gentamicin or kanamycin; 3 umoles of Tris HCl at pH 7.8; 0.3 umole of $MgCl_2$; and 6 umoles of 2-mercaptoethanol in a total volume of 70 ul. The mixture was incubated at 30C for 20 min, 20 ul was pipetted onto a 0.75 cm^2 piece of phosphocellulose paper (Whatman P-81). The papers were then immersed in hot double-distilled water (80C) for 2 min to stop the reaction, washed several times with large volumes of distilled water, dried at 37C and counted in a Beckman LS-250 liquid scintillator using a toluene scintillation fluid. Negative control reactions for non-specific binding [^{14}C] acetyl CoA or [^{14}C] ATP to the paper were done in the absence of gentamicin, this background incorporation of radioactive label was subtracted to give the actual values in the result.

Assay for phosphorylation of antibiotics was done according to Ozanne et al. (1969), except that the S-30 fraction was used in place of an osmotic shockate. The reaction mixture consisted of 20 ul of S-30 fraction, 0.5 umole of $MgCl_2$; 0.45 umole of γ- ^{32}P ATP; 0.005 umole of streptomycin or gentamicin; 1.5 umoles of Tris-HCl pH 7.8; and 0.03 umole of 2-mercaptoethanol in a total volume of 70 ul. The subsequent procedure was the same as described for the adenylating or acetylating assays, except that the phosphocellulose paper squares were pre-soaked in a solution of ATP (20 mM) and sodium pyrophosphate (0.1 M) to reduce background.

13. Purification of tritiated gentamicin.

Gentamicin was tritiated by the Radiochemical Centre, Amersham, using purified gentamicin supplied by Schering Corporation Limited. The product of the catalytic tritium-exchange was crude [^3H] gentamicin sulphate of which 19 mg contained 25 mCi. Purification was done using Sephadex G-10 gel filtration by the method described by Mahon et al., 1973. Sephadex G-10 (25 g) was suspended in 50 ml of 0.02% (w/v) aqueous NaCl, added to a column (15 x 30 cm; Pharmacia), and washed with 1 liter of 0.02% NaCl. Crude [^3H] gentamicin dissolved in 1 ml of 20% sucrose (w/v) was applied to the column and eluted with 0.02% NaCl at a flow rate of 0.4 ml/min. Fractions (0.8 ml) were collected and 5 ul aliquots of each fraction were pipetted onto paper discs (7 mm diameter). Dried discs were placed on Muller-Hinton agar plates previously spread with a lawn of P. aeruginosa strain 280 (approximately 2 x 10^7 organisms). Incubation was at 37C for 24 hours. The fractions that contained antimicrobial activity (zone size = 17 mm or greater) were pooled. Purity of the pooled fraction was examined

with paper chromatography utilizing the chloroform-methanol-ammonia solvent system. The procedure was carried out according to Wagman et al. (1968); upper phase solvent was placed in the bottom of a chromatographic jar 3 hours prior to use and the lower phase was used for descending chromatography of Whatman No. 1 paper for 5 hours. The paper was dried, cut into small pieces (1 x 2.5 cm) and counted in a Beckman LS 250 liquid scintillator using a toluene scintillation fluid. Antibacterial activity was also examined by a broth dilution technique using P. aeruginosa strain 280 as the test organism. A 5 ul amount of the final pool, whose activity was similar to that seen with a disc containing 10 ug/ml gentamicin was pipetted onto a 0.75 cm^2 of filter paper which was dried at room temperature and counted in a Beckman LS 250 scintillator using a toluene scintillation fluid.

14. Gentamicin uptake study.

The method used was modified from that of Tseng et al. (1972). A 10 ml amount of a slime-free overnight broth culture was inoculated into 200 ml of fresh broth and incubated at 37C with vigorous shaking until the absorbance at 600 nm was 0.25. Twenty ml aliquots were transferred to 125 ml Erlenmeyer flasks containing various concentrations of mixed labelled and unlabelled gentamicin. The specific activity of [^{3}H]-gentamicin used was 0.0563 uCi/ug for gentamicin concentration less than 2.0 ug/ml and 0.0035 uCi/ug for higher concentrations. Non-specific background binding of gentamicin was determined by incubating a 10 ml volume of each of these mixtures at 4C for 2 hours in the presence of 0.025% sodium azide (w/v). The remaining 10 ml of each mixture in the flasks was incubated at 37C in a water bath with vigorous shaking for 2 hours and the absorbance at 600

nm was determined. The bacteria were harvested by centrifugation at 4,300 g for 20 minutes and washed three times with cold TS broth by centrifugation at 12,100 g for 10 minutes using the JA-20 rotor in a Beckman J-21 centrifuge at 4C. The final pellet was suspended in 2 ml of distilled water, subjected to ultrasonic vibration with a Bronson Biosonik 111 (3/8" diameter) at 4C for 1 minute, dispersed in 10 ml of Bray scintillation fluid (60 g naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml absolute methanol, 20 ml ethylene glycol and P. dioxane to make 1 liter) and counted in a Beckman model LS-250 liquid scintillator.

15. Hot acid extraction of cell-associated gentamicin.

P. aeruginosa strain 8803 and strain 3503 were grown in 100 ml TS broth until the absorbances at 660 nm were 0.5. Sufficient labelled and unlabelled gentamicin were added to obtain a gentamicin concentration of 100 ug/ml for strain 8803 (specific activity = 0.0004 uCi/ug) and 0.625 ug/ml for strain 3503 (specific activity = 0.0225 uCi/ug). After two further hours of incubation at 37C, the bacteria were harvested and recovered after each of the three were washed with cold TS broth by centrifugation at 12,100 g for 10 minutes with JA-20 rotor in a Beckman J-21 centrifuge at 4C. The final pellet was resuspended in 3 ml of cold 6% perchloric acid (v/v) and then heated in boiling water for 5 minutes. A 25 ul sample was dispersed in 5 ml of Bray scintillation fluid and counted. The supernatant containing extracted gentamicin was obtained by centrifugation at 4,300 g. with the JA-20 rotor in a Beckman J-21 centrifuge for 15 minutes and a 25 ul sample was counted. From these two counts, the percentage of extraction could be calculated. Hot acid extracted gentamicin was then neutralized with 5N KOH, centrifuged at 12,100 g in a Beckman J-21

centrifuge for 10 minutes and the supernate (extracted gentamicin) was used for paper chromatography. Control specimens of ^{3}H-gentamicin and gentamicin were subjected to the hot acid treatment and examined for retention of antibiotic activity by a microbiological assay and for chemical change by paper chromatography.

16. Method confirming non-specific background binding ^{3}H-gentamicin to cells treated with NaN_3 (0.025%) and 4 C.

P. aeruginosa strains 3503 and 8803 were grown in 60 ml of TS broth until the absorbances at 600 nm were 0.25. Ten ml aliquots were transferred to five 125-ml Erlenmeyer flasks. Gentamicin was added to the MIC concentration for each strain (Table 111) to the second and fifth flask; gentamicin and 0.025% sodium azide (w/v) were added to the third flask. The first three flasks were incubated at 4C and the remaining flasks were incubated at 37C with vigorous shaking. After 2 hours incubation, the bacteria were harvested and recovered after each of three washes with cold TS broth by centrifugation at 12,100 g for 15 minutes at 4C. The final pellets were resuspended in 10 ml of TS broth. Volumes of 0.2 ml from appropriate dilutions were plated on TSA and incubated at 37C. After overnight incubation, viable colonies were counted.

RESULTS

1. Strain characterization and pyocine typing.

Fourteen strains of hospital-isolates of P. aeruginosa which were found to be resistant to gentamicin (Tables 111, V) were selected as representative strains for study of their mechanism of resistance to gentamicin. The characterization of these strains as P. aeruginosa is given in Table 1. Strain 280 GSK^r, a strain of P. aeruginosa trained to gentamicin resistance in the laboratory by stepwise repeated transfer in increasing gentamicin concentrations required further characterization. That strain had a prolonged generation time in TS broth, was auxotrophic (Tseng et al., 1972) and changed in several properties basic to the identification of P. aeruginosa. However, the DNA base ratio was identical to the parent strain 280 (66.5% G+C content) and a single polar flagellum was detected by electron microscopy. Thus although certain conventional properties associated with P. aeruginosa were lost by strain 280 GSK^r, it retained the basic metabolic characteristics of Pseudomonas (Table 1) and the other properties noted above which confirmed it to be a mutant of strain 280. Pyocine typing of strains (Table 11) showed that the strains comprised five different types and thus were extremely unlikely to be a single strain spreading throughout the hospital. Strains 14043, 13934, 8625, 14786 and 8803 were known to have originated from the same patient who was undergoing topical treatment with gentamicin.

2. Susceptibility tests.

Susceptibility tests to several aminoglycoside antibiotics were done by a broth dilution technique and by the Kirby-Bauer disc method, the results of which are shown in Tables 111 and V respectively. These

TABLE I Characterization of *P. aeruginosa* strains.

Strain	Motility	Growth in air	Catalase	Oxidase	Glucose (acid)	O-F test	Growth at 42 C*	Glucose oxidation	King medium A	King medium B	Growth TPTZ**	Acetamide hydrolysis	Arginine dihydrolase
280GSK^r	+	+	+	+	+	O+F-	+	-	-	-	-	-	-
8803	+	+	+	+	+	O+F-	+	+	+	+	-	+	+
1136	+	+	+	+	+	O+F-	+	+	+	+	+	+	+
8476	+	+	+	+	+	O+F-	+	+	+	+	+	+	+
14786	+	+	+	+	+	O+F-	+	+	+	+	+	+	+
11276	+	+	+	+	+	O+F-	+	+	+	+	+	+	+
2148	+	+	+	+	+	O+F-	+	+	-	+	+	+	+
996	+	+	+	+	+	O+F-	+	+	-	+	+	+	+
14043	+	+	+	+	+	O+F-	+	+	+	+	+	+	+
8625	+	+	+	+	+	O+F-	+	+	+	+	+	+	+
13934	+	+	+	+	+	O+F-	+	+	+	+	+	+	+
1444	+	+	+	+	+	O+F-	+	-	+	+	+	+	+
Pmu78	+	+	+	+	+	O+F-	+	+	+	+	+	+	+
10804	+	+	+	+	+	O+F-	+	-	+	+	+	+	+

* Three consecutive transfers.

** One percent triphenyltetrazolium chloride in nutrient agar No. I.

TABLE II. Pyocine typing of the gentamicin-resistant P. aeruginosa.

Strain	Type	Sub-type
280GSK^r	NT*	
8803	16	
1136	3	
8476	3	
14786	16	
11276	3	
2148	NT	
966	3	
14043	16	
8625	16	
13934	16	
1444	1	c
Pmu78	1	c
10804	12	

Pyocine typing was by the method of Gavan and Gillis (1969).

NT* : non-typable.

results show that the resistance range for gentamicin was from 6.25 ug/ml to 100 ug/ml. All of these strains would be regarded as clinically resistant. Strains grown in the presence of a low concentration of gentamicin(5ug/ml), underwent an increase in the MIC of gentamicin of from 2 to 4-fold. This observation was made because strains were grown in low gentamicin concentration to induce inactivating or other types of gentamicin modifying enzymes. The results are of interest because the gentamicin concentration used was near the maximal obtainable blood and tissue concentration. A similar emergence of a more resistant strain was seen during treatment of a patient with gentamicin (13934, 8803). The susceptibility results also demonstrate that most of the strains carry cross-resistance to several other new aminoglycoside antibiotics, some of which have not been used at the University of Alberta Hospital, Edmonton.

3. Mating system.

No transfer of gentamicin resistance could be detected by conjugation to three P. aeruginosa recipients and one E. coli recipient from the gentamicin-resistant strains. Three hour and overnight matings were used as well as a 3-hour mating and overnight incubation in drug-free medium. The limits of detection of the system are mating frequencies of $<5 \times 10^{-8}$ per donor cell.

4. Analytical CsCl gradient centrifugation.

The mating system used was developed by Bryan et al. (1972) and was found to be very sensitive as it could be used to detect a mating frequency as low as 10^{-8}. There are two possibilities which may account for the non-transferability; firstly, the transfer frequency for gentamicin resistance is too low to be detected. Bryan and co-

TABLE III. Cross resistance of P. aeruginosa strains to various aminoglycoside antibiotics.

Strain	MIC ug/ml			
	Gentamicin	Tobramycin	BB-K8	Sisomicin
280GSK^r	100	>100	50	>100
8803	100	50	>100	100
1136	50	25	>50	50
8476	50	25	>50	50
14786	50	25	>50	50
11276	50	25	>50	50
2148	25	12.5	>25	25
966	25	12.5	>25	25
14043	25	12.5	>25	25
8625	25	12.5	>25	25
13934	25	12.5	>25	25
1444	12.5	6.25	>12.5	12.5
10804	6.25	0.75	>12.5	1.5
Pmu78	6.25	0.75	12.5	0.75
280*	0.25	0.375	0.75	0.15

* Sensitive strain.

TABLE IV. The MIC of P. aeruginosa before and after growth in the presence of gentamicin (5 ug/ml) for seven days.

Strain	MIC (gentamicin ug/ml)	
	Before	After
280GSK^r	100	>400
8803	100	200
1136	50	100
8476	50	100
14786	50	100
11276	50	100
2148	25	50
966	25	100
14043	25	100
8625	25	100
13934	25	100
1444	12.5	50
10804	6.25	>25
Pmu78	6.25	20

Cultures were transferred daily to new TS broth containing 5 ug/ml gentamicin.

TABLE V. Resistance characteristic of strains of P. aeruginosa determined by disc susceptibility testing.

Strain	Resistance characters								
	$TH_{1.0}$	Te_{30}	S_{10}	CB_{100}	K_{30}	Gent	BB_{10}	Sis_{10}	T_{10}
$280GSK^r$	+	-	+	-	+	+	+	+	+
8803	+	+	+	-	+	+	+	+	+
1136	+	+	+	-	+	+	+	+	+
8476	+	+	+	-	+	+	+	+	+
14786	+	+	+	-	+	+	+	+	+
11278	+	+	+	-	+	+	+	+	+
2148	+	+	+	-	+	+	+	+	+
966	+	+	+	-	+	+	+	+	+
14043	+	+	+	-	+	+	+	+	+
8625	+	+	+	-	+	+	+	+	+
13934	+	+	+	-	+	+	+	+	+
1444	-	+	+	-	+	+	+	+	+
10804	-	+	inter	-	+	+	-	-	-
Pmu78	+	+	+	-	inter	+	-	-	-

$TH_{1.0}$: Thiosulfil 1.0 mg disc.
Te_{30} : Tetracycline 30 ug disc.
S_{10} : Streptomycin 10 ug disc.
CB_{100} : Carbenicillin 100 ug disc.
K_{30} : Kanamycin 30 ug disc.
Gent : Gentamicin 10 ug disc.
BB_{10} : BB-K8 10 ug disc.
Sis_{10} : Sisomicin 10 ug disc.
T_{10} : Tobramycin 10 ug disc.
inter : intermediate.
+ : resistant
- : sensitive

workers (Bryan et al., 1973) found that the transfer frequency for gentamicin resistance was 10 to 100-fold lower than that obtained for streptomycin resistance. Secondly, these gentamicin-resistant P. aeruginosa strains may contain antibiotic resistance determinants on plasmids which are non self-transmissible. Extracts of such strains may be run in an ultracentrifuge using a CsCl density gradient to ascertain whether they contain any extrachromosomal DNA.

Densitometer traces of photographs taken during CsCl gradient centrifugation from strain 1136 showed only a single peak, chromosomal DNA (Figure 7b). Strains 8803 and 13934 showed a satellite peak in addition to the major peak of chromosomal DNA (Fig. 7a,c). The characteristics and functions of this extrachromosomal DNA, are currently unknown and are being investigated in our laboratory.

5. Inhibition of amino acid incorporation by gentamicin.

The aminoglycoside antibiotics exert their antimicrobial effect by irreversible inhibition of protein synthesis in susceptible cells. In the case of streptomycin, it has been shown that the drug acts by binding to the 30S ribosomal subunit and interferes with the functions of the A site of the ribosome. Streptomycin resistance has been characterized as a mutation affecting the 30S ribosome subunit in bacteria, and studies have shown that streptomycin resistance is the result of a single amino acid change in a protein (S_{12}) of the small subunit. This alteration prevents the binding of the drug. Similarly, gentamicin inhibits protein synthesis in susceptible cells. Gentamicin resistance could be mediated by a ribosomal or other mutation in the protein synthesizing components of the cell. Thus, it is necessary to study in vitro protein synthesis in these strains and the effect of gentamicin

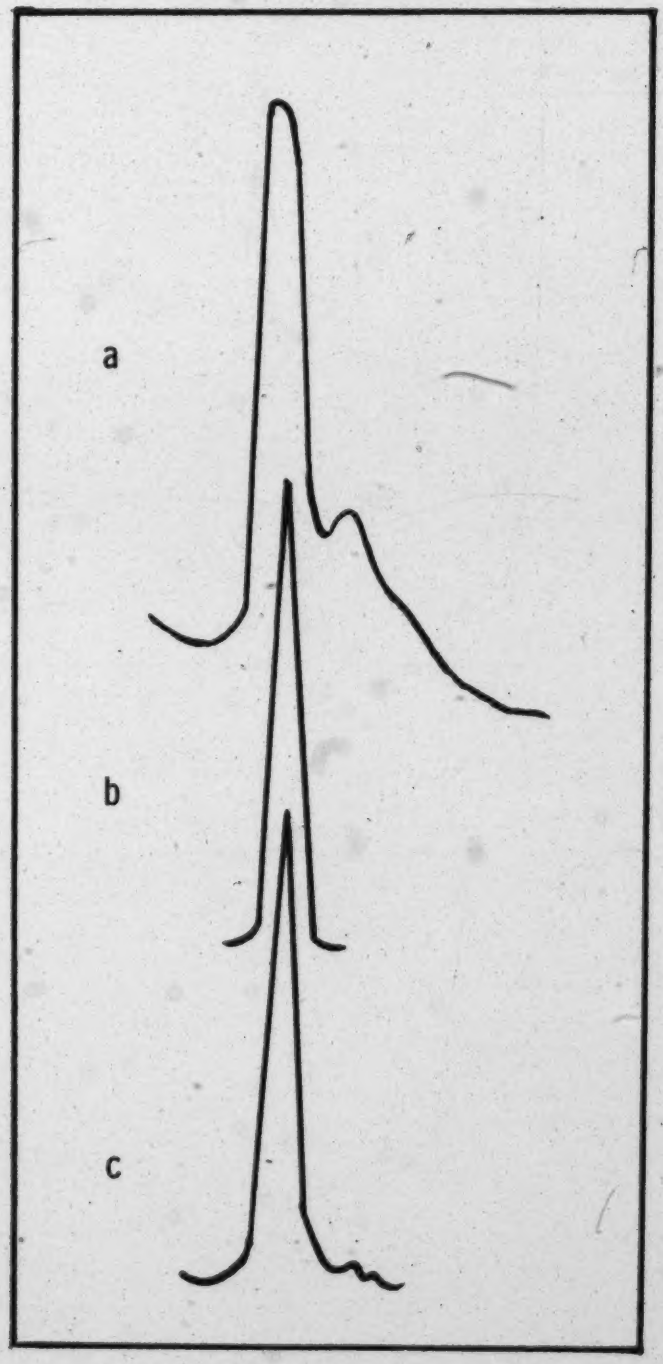

a
b
c

FIGURE 7: Densitometer Traces of DNA from P. aeruginosa strains.

Trace a : DNA from strain 8803

Trace b : DNA from strain 1136

Trace c : DNA from strain 13934

on that process.

The results of in-vitro protein synthesis are shown in Tables VI and VII. Tables VI and VII illustrate that ribosomes of all strains studied were sensitive to gentamicin. Some of these strains were also sensitive to tobramycin (Table VII). When the S-100 fraction of strain 8803 was used as a source of soluble components for protein synthesis in strain 8803 instead of the S-100 fraction of sensitive strain 280, protein synthesis was still inhibited by gentamicin (Table VII). It can be concluded that gentamicin inhibits protein synthesis of these gentamicin-resistant P. aeruginosa strains in vivo.

6. Enzymatic inactivation of gentamicin.

In natural isolates, the principal mechanism of resistance to antibiotics results from the presence of enzymes that specifically modify the antibiotic so that it can no longer interact with its target in the cell. These enzymes inactivate aminoglycoside antibiotics by acetylation of amino groups, phosphorylation of hydroxyl groups, or adenylation of hydroxyl groups. A distinguishing characteristic of these strains is that the genes determining the inactivating enzymes are usually carried on extrachromosomal elements which exist in the resistant strains. These extrachromosomal elements are known as R-factors. In the case of gentamicin, it has been shown that the drug can be inactivated by kanamycin acetyltransferase, gentamicin acetyltransferase 1, gentamicin acetyltransferase 11 or gentamicin adenyltransferase.

The enzymatic inactivation of gentamicin was studied by three different methods.

A. Microbiological Assay. No detectable reduction in anti-

TABLE VI. Effect of gentamicin on the incorporation of ^{14}C valine by cell-free systems directed by R17RNA.

System		uumoles incorporated in 250 ul		% inhibition
Ribosome	S 100	- gentamicin	+ gentamicin	
8803	280	19.8	6.6	66.4
1136	280	73.4	46.3	36.9
8476	280	47.2	35.9	23.8
14786	280	48.4	37.3	22.8
11276	280	36.1	31.1	13.6
2148	280	84.4	37.3	55.7
966	280	93.1	43.7	53.0
14043	280	68.4	38.6	43.5
8625	280	83.8	38.1	54.5
13934	280	28.4	10.5	62.8
1444	280	26.2	20.2	22.9
280	280	35.9	16.4	54.2

All strains are P. aeruginosa. The numbers listed under ribosome are the strain numbers from which ribosomes were obtained. The S 100 fraction was in all cases obtained from the sensitive strain, 280.

TABLE VII. Effect ofgentamicin on the incorporation of ^{14}C phenylalanine by cell-free systems directed by poly U.

System		uumoles incorporated in 250 ul.		% inhibition	uumoles incorporated in 250 ul. + tobramycin	% inhibition
Ribosome	S100	- gent	+ gent			
280GSK^r	280	421.3	54.1	87.1	29.6	92.9
8803	280	58.2	25.2	56.7	-	-
1136	280	73.9	23.7	67.8	29.7	59.7
8476	280	256.7	148.9	42.0	-	-
14786	280	266.9	107.0	59.8	-	-
1444	280	225.4	88.8	60.5	-	-
11276	280	80.9	31.5	61.0	26.1	67.8
2148	280	69.9	22.5	67.8	-	-
966	280	99.6	30.8	69.0	-	-
14043	280	311.5	104.5	66.4	-	-
8625	280	87.1	19.3	77.7	12.3	85.8
13934	280	67.1	20.9	68.8	16.2	75.9
Pmu78	280	33.6	16.9	49.6	15.5	53.8
10804	280	47.7	32.7	31.5	14.8	69.0
280	280	60.8	26.3	61.7	-	-
8803	8803	68.8	36.7	46.6	-	-

All strains are P. aeruginosa. The numbers listed under ribosome are the strain numers from which ribosomes were obtained. Similarly, the numbers listed under the S100 fraction are the strain numbers from which the S100 fractions were obtained.

bacterial activity of gentamicin occurred when the drug was mixed with cell-free extract (S-30 fraction) of resistant strains in the presence of acetyl coenzyme A or adenosine triphosphate.

This assay has one basic limitation: it can only measure the modification of a substance that is an antibiotic, and any potential modifications that do not result in significant inactivation of the antibiotic would not be detected.

B. Chemical Assay. Results shown in Table VIII indicate that gentamicin was not inactivated using cell-free extracts of strain 8803, 1136 and 13934 by acetylation, adenylation or phosphorylation. The cpm/mg protein values of these strains were similar to that of strain 280 which is sensitive to gentamicin.

P. aeruginosa 130 contains gentamicin acetyltransferase 1 that will, in the presence of acetyl coenzyme A transfer an acetate moiety to gentamicin. Gentamicins are strongly basic compounds and thus bind to cation exchange paper (phosphocellulose). A high cpm/mg protein value was obtained in the chemical assay using a P. aeruginosa 130 extract as shown in Table VIII. Similarly, P. aeruginosa POW 151 contains gentamicin adenyltransferase that will, in the presence of ^{14}C ATP, transfer an adenyl (AMP) group to gentamicin. P. aeruginosa 931 contains streptomycin phosphotransferase which, in the presence of γ-^{32}P ATP, transfers γ-^{32}P to streptomycin. This assay was carried out as a positive control of the phosphorylation assay. It is known that gentamicin C complex cannot be inactivated by phosphorylation, (although it can be phosphorylated) because of the lack of 3'-hydroxyl group of the purpurosamine ring. Table VIII also confirms that gentamicin was not inactivated by phosphorylation.

TABLE VIII. Enzymatic inactivation assay of aminoglycoside antibiotics by *P. aeruginosa* strains.

Strain	Resistance characters			cpm/mg. protein/ml				
				Acetylation		Adenylylation	Phosphorylation	
	Gent.	Kan.	Str.	Gent.	Kan.	Gent.	Gent.	Str.
130	+	+	+	5880.6	ND*	ND		ND
POW151	+	+	+	ND	ND	1068.5		ND
280/RPOW151	+	-	+	ND	ND	1955.0		ND
931	-	+	+	ND	ND	ND	346.0	15800
280	-	-	-	15	ND	25.8	209.5	ND
8803	+	+	+	0	15	15.0	165.0	ND
1136	+	+	+	2	10	10.0	239.4	ND
13934	+	+	+	17.5	2.5	12.5	217.2	ND

*ND = not done, + = resistant, - = sensitive.

C. Microbiological assay and paper chromatography of hot acid extracted gentamicin from strains 8803 and 3503. The percentages of extraction of gentamicin from strains 8803 and 3503 by hot acid were 99 and 88.3 respectively, based on the recovery of tritium label. Table IX shows that the hot acid extracted gentamicin from strains 8803, 3503 and standard gentamicin which was subjected to the hot acid treatment showed reduced antibiotic potency. However, the gentamicin extracted from resistant strain 8803 was at least as potent as that extracted from the sensitive strain 3503.

Cell-associated gentamicin extracted from P. aeruginosa strain 8803 subjected to paper chromatography showed three radioactive peaks (Fig. 8) similar to those obtained with hot acid treated standard tritiated gentamicin (Fig. 9). The relative amount of the C_2 gentamicin component, however, was reduced. Gentamicin similarly extracted from sensitive strain 3503 and chromatographed also shows a reduction in C_2 component suggesting less of that component penetrates P. aeruginosa cells. The evidence presented suggests that strain 8803 does not modify or inactivate gentamicin by an unknown mechanism.

7. Purification of tritiated gentamicin.

Aliquots (5 ul) of fractions from a Sephadex G-10 column used to purify ^{3}H-gentamicin were applied to paper discs and these in turn to a lawn of P. aeruginosa strain 280. Fractions 14 to 19 gave a zone of inhibition of 17 mm or greater and were pooled, producing a total volume of 4.8 ml. Gentamicin was quantitated by comparing zone sizes of standard gentamicin discs with discs containing aliquots of the pooled gentamicin solution. A 5 ul amount of pooled hot gentamicin contained 10 ug of gentamicin and the total radioactivity of the 4.8 ml

TABLE IX. Microbiological assay of hot acid extracted gentamicin by a broth dilution technique using P. aeruginosa strain 280 as the test organism.

	ug/ml gentamicin							
	1	0.5	0.25	0.125	0.06	0.03	0.015	0.007
Extracted gentamicin from 8803	-	-	+	+	+	+	+	+
Extracted gentamicin from 3503	-	+	+	+	+	+	+	+
Hot acid treated gentamicin	-	-	-	+	+	+	+	+
Standard gentamicin	-	-	-	-	+	+	+	+

- is no growth, + indicates growth.

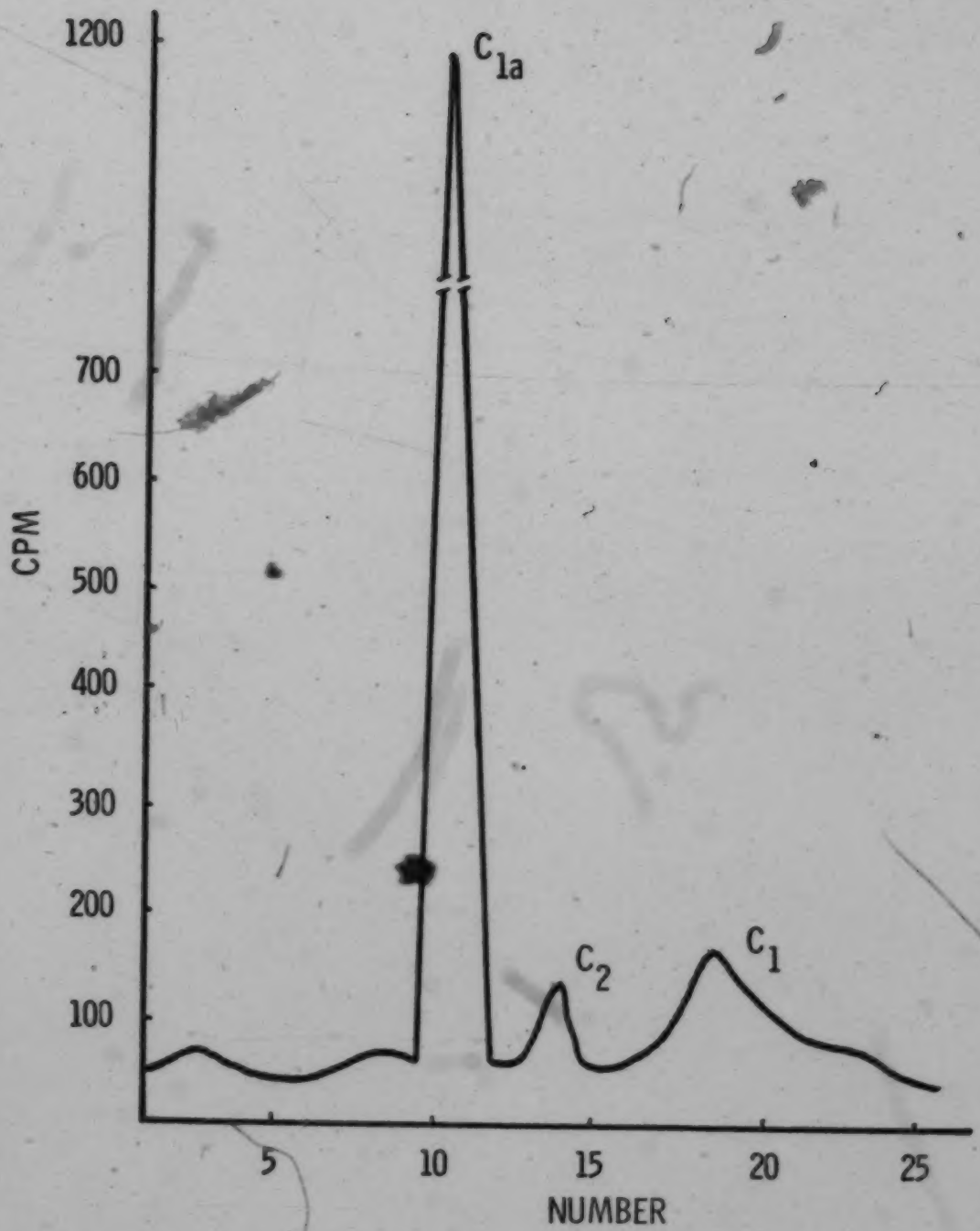

1200
700
600
500
400
300
200
100
CPM
C_{1a}
C_2
C_1
5
10
15
20
25
NUMBER

FIGURE 8: Radiochromatograph of hot acid extracted gentamicin from *P. aeruginosa* strain 8803.

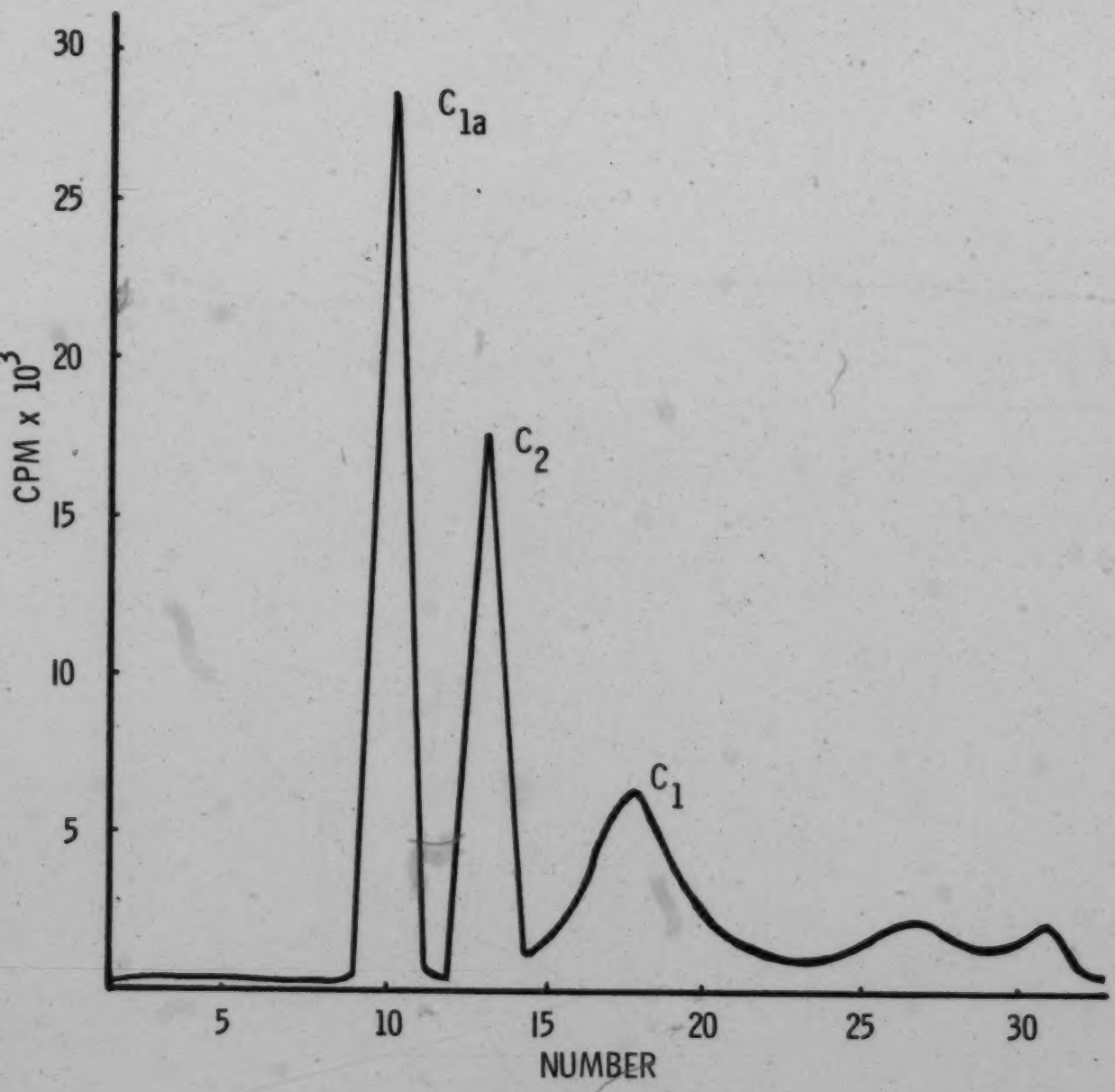

30
25
20
15
10
5
CPM x 10^3
C_{1a}
C_2
C_1
5
10
15
20
25
30
NUMBER

FIGURE 9: Radiochromatograph of hot acid treated tritiated gentamicin.

pooled volume was 3×10^9 dpm. By assuming that the molecular weight of gentamicin was 463, (C_1 = 477, C_2 = 463, C_{1a} = 449) the specific activity could be calculated. It had a specific activity of 65.25 uCi/u mole. This purified hot gentamicin showed three radioactive peaks on the radiochromatogram corresponding to the three gentamicin components (C_{1a}, C_2 and C_1, respectively). There was no detectable impurity in the radiochromatograms (Fig. 10).

8. Gentamicin uptake.

The gentamicin-resistant hospital-isolates of P. aeruginosa strains studied contained no evidence of a gentamicin-inactivating enzyme and ribosomes from each of the strains were susceptible to gentamicin. These findings suggested the most probable mechanism of gentamicin resistance may be diminished permeability to the drug. Therefore, gentamicin uptake studies were carried out to assess this possibility.

The results are shown in Figures 11 and 12, Tables X1 and X11. The kinetics of uptake of ^{3}H-gentamicin with time by P. aeruginosa strain 3503 at 0.625 ug/ml gentamicin (the MIC of strain 3503) is shown in Figure 11. In the absence of 0.025% NaN_3 (w/v), there is an increase with time in the amount of ^{3}H-gentamicin associated with strain 3503; in the presence of 0.025% NaN_3 (w/v) at 4C the increase in gentamicin accumulation with time by P. aeruginosa strain 3503 is almost totally eliminated. Thus the cell-associated radioactivity obtained with 0.025% NaN_3 at 4C represents background level which appears to be non-specific binding of gentamicin to the bacteria. This interpretation was favored by the results of viable counts of strains 3503 and 8803 in the presence of gentamicin or gentamicin with sodium azide at 4C or 37C (Table X). If the survival of strain 3503 and 8803 cells with gentamicin alone is

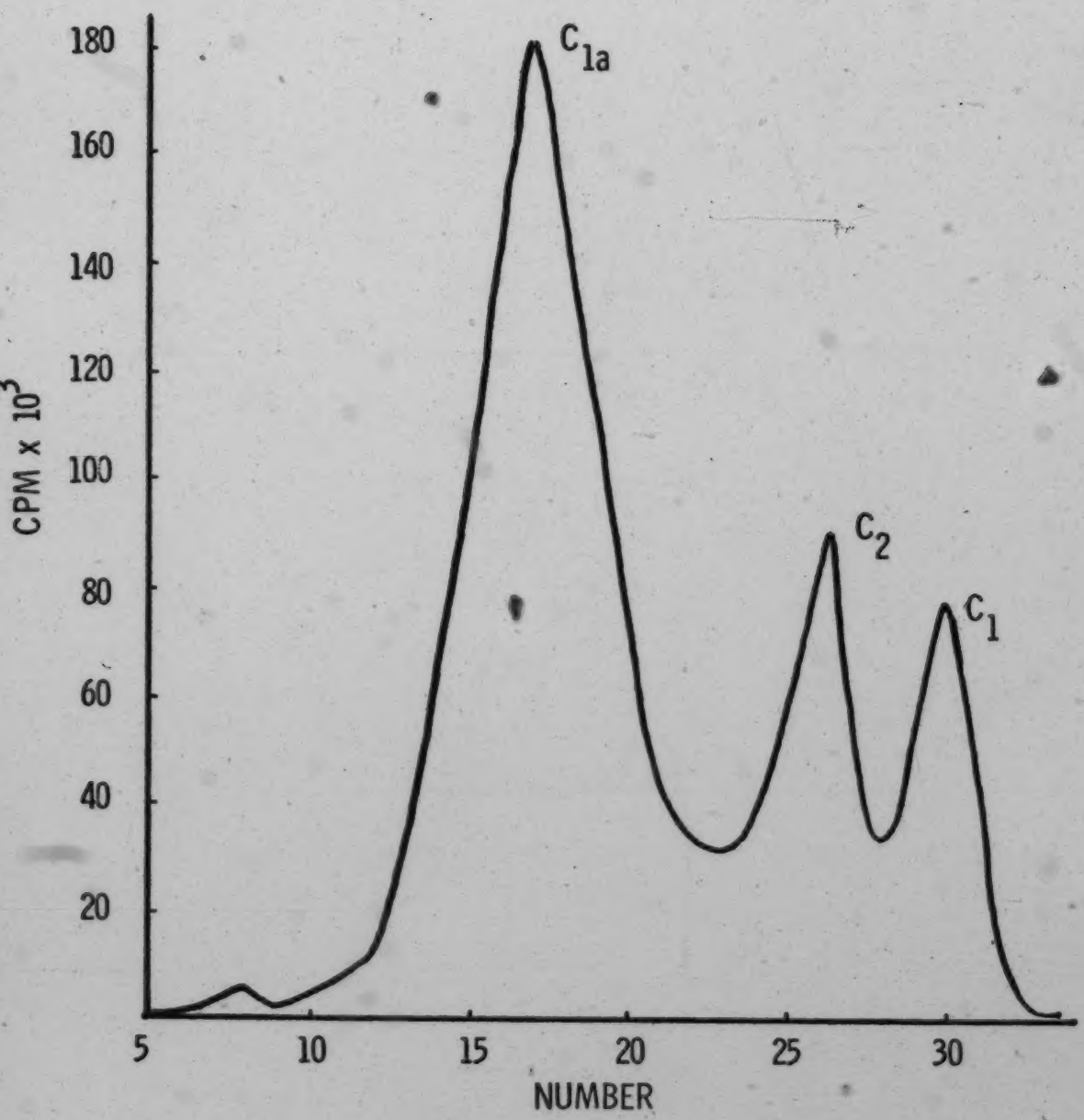

180
160
140
120
100
80
60
40
20
CPM x 10^3
C_{1a}
C_2
C_1
5
10
15
20
25
30
NUMBER

FIGURE 10: Radiochromatograph of tritiated gentamicin.

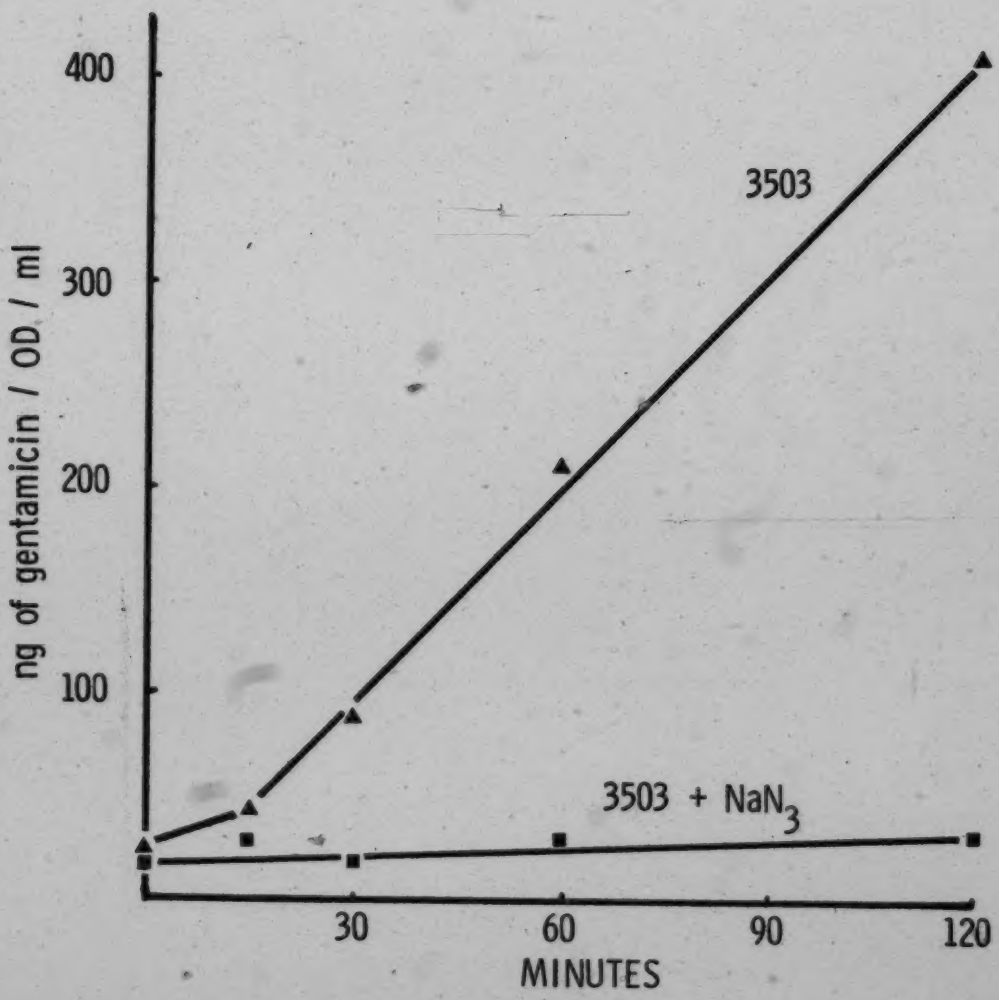

400
300
200
100
ng of gentamicin / OD / ml
3503
3503 + NaN3
30
60
90
120
MINUTES

FIGURE 11: Kinetics of uptake of gentamicin by strain 3503 in TS broth at a gentamicin concentration of 0.625 ug/ml. OD is an optical density unit at 600 nm.

TABLE X. Effect of gentamicin and sodium azide on *P. aeruginosa* 3503 and 8803 at various temperatures.

Strain	MIC ug/ml	Gentamicin concentration ug/ml	Temperture	Viable count (organism/ml)		
				Gentamicin	Gentamicin + NaN_3	Standard control
3503	0.625	0.625	4	2.5×10^8	2.15×10^8	3.85×10^8
3503	0.625	0.625	37	2.3×10^5	8.00×10^8	2.40×10^9
8803	100.000	100.000	4	2.4×10^8	2.70×10^8	2.00×10^8
8803	100.000	100.000	37	1.3×10^8	1.00×10^9	1.92×10^9
8803	100.000	200.000	37	1.0×10^7	5.90×10^8	1.52×10^9

Strains were grown in the presence of different concentrations of gentamicin and gentamicin with 0.025% (w/v) sodium azide for two hours at 4C and 37C. The bacterial cells were harvested and washed three times with cold TS broth. The final pellets were resuspended in 10 ml. of TS broth and serial dilutions plated on nutrient agar plates. Colony counts were done after overnight incubation at 37C. Strains exposed to 0.025% (w/v) sodium azide only as described above had cell counts essentially identical to those listed for gentamicin + NaN_3.

compared to that with gentamicin and 0.025% NaN_3 at 37C, a clear protective effect of sodium azide is demonstrated. This effect is even more pronounced if a comparison of cell survival in gentamicin is made to standard control counts which contained no gentamicin for the treatment period at 37C. The relative survival is less than 1% in all cases. Sodium azide prevents growth of cells and control counts of preparations containing only 0.025% sodium azide were essentially identical to those shown in Table X for preparations containing gentamicin and sodium azide. Low temperature is also protective. It could be concluded that there was little or no gentamicin penetration into the bacterial cell in the presence of sodium azide particularly at 4C and the method used to assess the non-specific background binding of gentamicin in the uptake study was correct. It also indicates that significant gentamicin penetration to the target site, the ribosome, does not occur in the presence of sodium azide. Therefore measurement of azide-sensitive gentamicin uptake appears to reflect actual intracellular drug accumulation. For the susceptible strain 3503 (MIC 0.625 ug/ml), the uptake was biphasic at 0.625 ug of gentamicin/ml (Figures 11, 12). The initial flat portion of the curve or primary uptake was very rapidly achieved, being essentially complete by the time the first sample was taken (0 minute sample). The second phase of uptake was linear and occurred after 15 minutes. The kinetics of ^{3}H-gentamicin uptake by the susceptible strain P. aeruginosa were similar to streptomycin uptake reported by Anand et al. (1960) and Tseng et al. (1972). The primary uptake was not azide-sensitive (Figure 11) and was considered to be physical adsorption on the cell surface since azide could significantly reduce killing of strain 3503 by gentamicin. The second

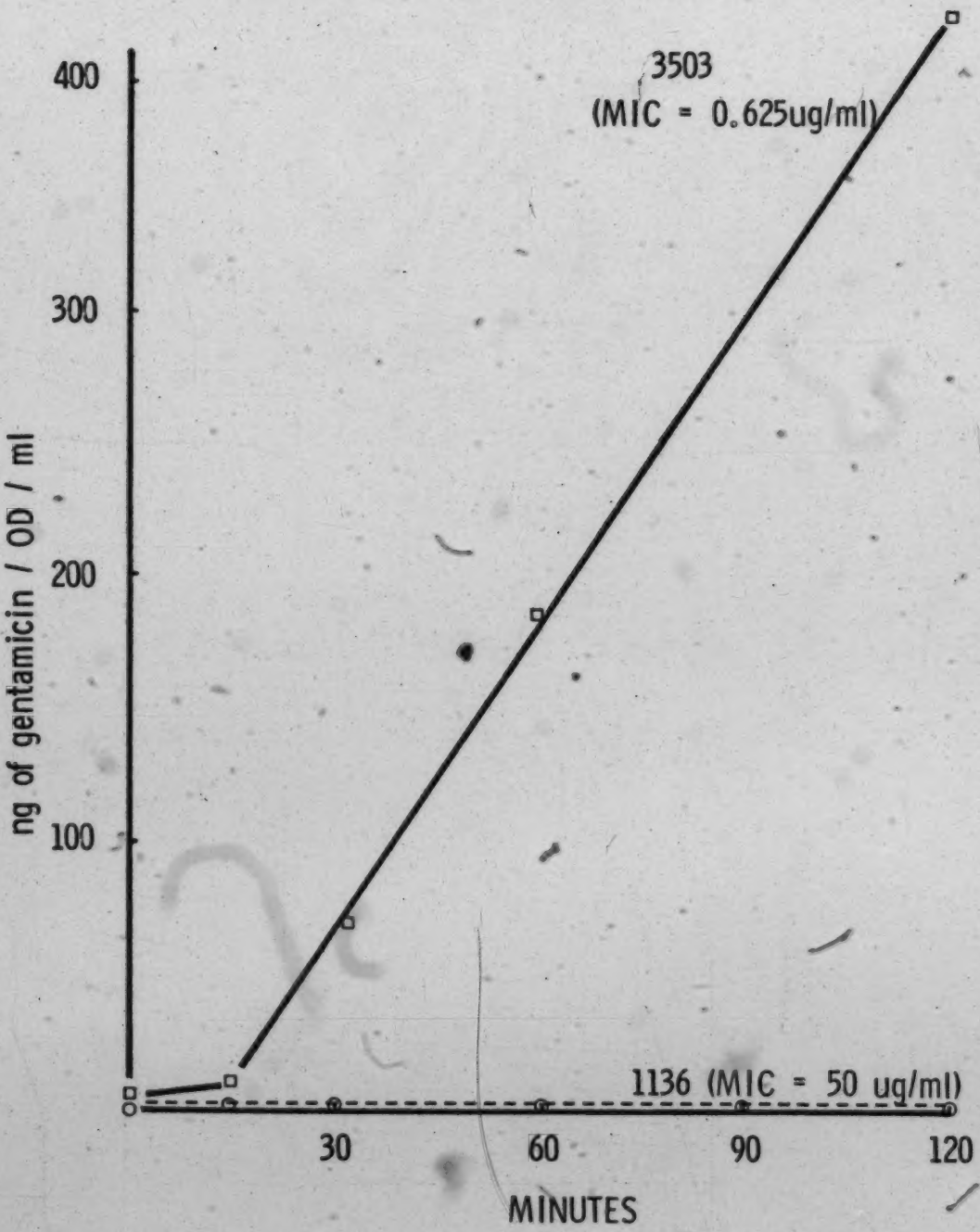

400
300
200
100
ng of gentamicin / OD / ml
3503
(MIC = 0.625ug/ml)
1136 (MIC = 50 ug/ml)
30
60
90
120
MINUTES

FIGURE 12: Kinetics of uptake of gentamicin by strains 3503 and 1136 in TS broth at a gentamicin concentration of 0.625 ug/ml.
OD is an optical density unit at 600 nm.

phase of gentamicin uptake was inhibited by sodium azide (Figure 11), a respiratory inhibitor, which interrupts the electron transport chain at cyt. C $Fe^{++} \rightarrow O_2$. Thus the second phase of gentamicin uptake appears dependent on the electron transport system. This pattern of gentamicin uptake was not seen with the resistant strain 1136 at 0.625 ug of gentamicin/ml (Figure 12).

For the susceptible strain 1310 and R^+ strain 1310 (R130), the uptake of gentamicin is independent of the MIC values (Table XI). The comparative uptakes of strains of 1310 and 1310 (R130) indicates that azide-sensitive uptake is not dependent on inhibition of protein synthesis nor likely on ribosomal binding of gentamicin. The strain 1310 (R130) is isogenic with strain 1310 except that it contains the R-factor R130. That R-factor specifies acetylation of gentamicin and causes gentamicin to be inactive as an antibiotic. Thus inhibition of protein synthesis does not occur until a concentration of 100 to 200 ug/ml gentamicin in the growth medium. From Table XI it is clear that although uptake is reduced when compared to strain 1310 it does occur to a significant extent and also occurs at 0.625 to 1.25 ug/ml, the concentration at which significant gentamicin begins in strain 1310. Although not yet rigorously proven, it is highly likely that gentamicin acetate does not bind to a primary site on the ribosome, unlike gentamicin. This has been shown to be true for streptomycin adenylate, streptomycin phosphate and lividomycin phosphate. A radioautography study (Shahrabadi, 1974, personal communication) demonstrates that gentamicin can be shown in an intracellular location in the R^+ or R^- strain. This further supports the contention that penetration of gentamicin occurs even in the absence of inhibition of protein

synthesis. Thus the accumulated data indicates that gentamicin uptake is not dependent upon some event which occurs at the MIC as uptake and MIC can be divorced. The most likely event would be inhibition of protein synthesis from which azide-sensitive uptake seems quite independent.

Resistant strain 1136 (MIC 50 ug/ml) accumulated much less gentamicin than did the susceptible strain 3503 at 0.625 ug/ml of gentamicin (Figure 12). Table XI presents gentamicin uptake data in TS broth at different gentamicin concentrations by representative strains of P. aeruginosa. These strains show an extremely close relationship between the MIC and significant uptake of gentamicin. The gentamicin uptake of the resistant strains as demonstrated by strains 8803, 1136 and 13934 show little or no gentamicin incorporation occurred below the MIC. A similar observation was noted by Tseng et al. (1972) in streptomycin resistant P. aeruginosa. The pattern of uptake of gentamicin and streptomycin by strain 280 GSK^r appears to be similar. Hence we can conclude that gentamicin resistance in P. aeruginosa is likely due to reduced permeability to the drug. A gentamicin uptake study was also done with strain 13934 after growth in the presence of a low concentration of gentamicin (5 ug/ml) for 7 serial transfers. Under these conditions the MIC of strain 13934 increased by 4-fold from 25 ug/ml to 100 ug/ml (Table IV). Table XII shows that the uptake of gentamicin by strain 13934 after the serial transfers in gentamicin declined by 100% when compared to the same strain not subject to this treatment. This finding again supports a relationship between gentamicin permeability and resistance to gentamicin.

9. Summary.

TABLE XI. Actual uptake of gentamicin by strains of P. aeruginosa at various gentamicin concentrations.

Strain	MIC ug/ml	Actual uptake (ng/A600 unit/ml)*										
		Gentamicin concentration (ug/ml)										
		0.3125	0.625	1.25	2.5	5.0	10.0	25.0	50.0	75.0	100	125
3503	0.625	11.0	170.0[a]	220.0	420.0	905.0	2727.4					
1310	1.6	2.67	26.7	330.2[a]	672.2	1380.0						
1310R130[b]	100.0		59.1	61.2	135.5	460.3		1336.0	3022.8	5928.6	9987.0[a]	10733.4
280GSK[r]	100.0								0	0	158.3[a]	237.2
8803	100.0	0	0	0	0	0	0	0	0	0	820.7[a]	
1136	50.0	0	0	0	0	0	0	0	471.1[a]			
13934	25.0	0	0	0	0	0	0	132.1[a]				

* The actual uptake was calculated by substracting the non-specific background binding of the drug from the total uptake and given as nanograms of gentamicin per A600 unit of growth per ml.

a : Values underlined represent gentamicin uptake at or about the MIC.

b : Strain grown in the presence of 10 ug/ml gentamicin for five days underwent an increase in the MIC of gentamicin of from 25ug/ml to 100 ug/ml.

TABLE XII. Actual uptake of gentamicin by P. aeruginosa 13934 and induced-resistant P. aeruginosa 13934 at a gentamicin concentration of 30 ug/ml.

Strain	MIC (ug/ml)	Actual uptake ng/10^9 cells	% decrease of uptake
13934	25	97.6	
13934*	100	0	100

* Induced-resistant P. aeruginosa 13934

Strain 13934 was serially transferred in TS broth containing 5 ug/ml gentamicin for seven days.

The gentamicin uptake data show that there is a close correlation between a strain's MIC and its uptake value. The ribosomes are sensitive to gentamicin in the inhibition of amino acid incorporation in the cell-free system studied. No inactivating enzymes and R-factors were detected. From these findings, it has been concluded that resistance to gentamicin in P. aeruginosa is due to defective permeation of the drug.

DISCUSSION

The five different pyocine-type of P. aeruginosa used were hospital isolates which are a good representation of the resistant strains of P. aeruginosa occuring at the University of Alberta Hospital in Edmonton. The spectrum of susceptibility of these to gentamicin covers a range of MIC values from 6.25 ug/ml to 100 ug/ml. Most if not all of these strains would be regarded as clinically resistant since a dose of 1.5-8 mg/kg per day of gentamicin gives serum concentration of 2-16 ug/ml (Riff and Jackson 1971). However dose levels above 7-8 ug/ml are regarded as potentially toxic.

The ribosomes obtained from these strains are susceptible to gentamicin in the inhibition of in-vitro protein synthesis directed by both bacteriophage R17 RNA and poly U. No description of ribosomes resistant to gentamicin in any strains of bacteria has been reported at this time.

There is no evidence of gentamicin inactivation by acetylation, adenylation or phosphorylation. From the result reported (Table VII), substitution of the S-100 fraction of strain 8803 did not eliminate the effect of gentamicin on protein synthesis. Thus, the soluble components necessary for amino acid incorporation were not affected by gentamicin. The only added components to in-vitro amino acid incorporation not from the resistant strain were tRNA and poly U. No known mechanism of modification of these substances by any antibiotic has been reported.

The addition of the S-100 fraction to the amino acid incorporation studies further supports the contention that no inactivating enzymes are present in strain 8803. The amino acid incorporation system contains many of the co-factors necessary to inactivate gentamicin

should an inactivating enzyme have been present. The absence of such enzymes was also demonstrated by microbiological and radio-active assays. Additionally, extracted cell-associated gentamicin was chromatographed and followed a pattern similar to that of control standards or gentamicin extracted from sensitive strains. A combination of these findings make it exceedingly unlikely that gentamicin is inactivated.

Analytical CsCl gradient centrifugation of DNA shows extra-chromosomal peaks in strain 8803 and 13934 which are plasmid DNA, the sizes and functions of which are being determined in our laboratory. They could be plasmids that determine resistance to antibacterial substances although the lack of inactivating enzymes does not support this possibility. If they specify resistance, they apparently lack the transfer function of a complete R-factor and would represent resistance determinants. Mobilization of these resistance determinants by conjugation with a laboratory strain carrying a transfer factor and a recipient carrying neither a transfer factor nor a resistance determinant will support this conclusion but has so far failed. This conclusion could also be confirmed by transformation or by transduction with a bacteriophage of the plasmid DNA. Hybridization of the plasmid DNA from strains 8803 and 13934 with various P. aeruginosa R-factors is another possible way to study the function of these agents.

No genetic evidence of transferable gentamicin resistance could be detected in any of the strains despite careful attention to the conjugation procedure and the use of many different recipients. Extra-chromosomal DNA in strains 8803 and 13934 for the present is of unknown nature and must be termed cryptic plasmids. Plasmids with other

functions have been described in Pseudomonas such as the sex-factor termed, FP factors (Holloway 1969), and CAM plasmids which mediate degradation of camphor (Rheinwald et al 1973). Shahrabadi et al (Shahrabadi M.S., Bryan L.E., and Van Den Elsen H.M. manuscript submitted for publication) have also shown a cryptic plasmid in P. aeruginosa 931.

The kinetics of uptake of ^{3}H-gentamicin with time in a sensitive P. aeruginosa strain 3503 at a gentamicin concentration of 0.625 ug/ml (its MIC) showed that gentamicin uptake was apparently dependent on electron transport since it could be inhibited by sodium azide. Uptake was biphasic similar to that of streptomycin described by Anand et al (1960) in E. coli. The primary uptake was very rapidly achieved. In the presence of 0.25% (w/v) sodium azide, the uptake of gentamicin was approximately equal to the primary uptake. Thus, the primary uptake is most likely non-specific binding of the cationic gentamicin to the bacterial cells (see section 8 results). In the first 15 minutes the gentamicin uptake only slightly increased. There was then a secondary rise, levelling off after two hours at a two hundred-fold increase. This second phase of uptake occurred with strain 3503, but was not seen with resistant strain 1136 at 0.625 ug/ml of gentamicin. There was no gentamicin incorporation by strain 1136 at this concentration. From the results of these and earlier experiments it is likely that the resistance to gentamicin was due to diminished permeability of the drug. There were at least five lines of evidence which support that conclusion. Firstly, there was an extremely close correlation between the MIC and the actual uptake of gentamicin. The uptake values obtained for the sensitive strain 3503 were not achieved in resistant strains

8803, 1136 and 13934 until the gentamicin concentration approximated the MIC of the respective strains. The gentamicin uptake pattern of resistant strains were similar to that of strain 280 GSK^r, a mutant which has been shown by Tseng et al (1972) to have a permeability defect towards streptomycin. These patterns are also similar to streptomycin uptake of streptomycin-resistant P. aeruginosa whose mechanism of resistance is reduced permeability to streptomycin. The actual uptake values of gentamicin at the MIC concentrations of both sensitive and resistant strains were shown to reach target site in the cells since the drug could inhibit growth of those bacteria.

Secondly, when the gentamicin-resistant strains are grown in TS broth at a low concentration of gentamicin, there is a striking increase in resistance as shown in table IV. A similar phenomenon occurs in vivo in gentamicin-resistant strains isolated from the patient. This type of resistance was associated with decreased accumulation of the drug. Similar observations have been reported for E. coli (Franklin, 1967), Staphylococcus aureus (Sompolinski et al 1970) and P. aeruginosa (Tseng et al 1973) in tetracycline resistance.

Thirdly, the gentamicin uptake of strain 1310 (R130) which contains gentamicin-acetyltransferase, an enzyme inactivating gentamicin by acetylation is similar to that of strain 1310 in that uptake begins in both strains at the MIC at the R$^-$ strain. Thus although the R-factor results in a decrease in total uptake the concentration of gentamicin necessary to initiate uptake is the same for both strains. Therefore the correlation of uptake and MIC is not altered by the presence of another mechanism of resistance.

Fourthly, these resistant strains are also resistant to several

other aminoglycoside antibiotics (streptomycin, kanamycin, sisomicin, BB-K8 and tobramycin). There is no known single inactivating enzyme that can inactivate all of them. The ribosomes of some of these resistant strains are sensitive to tobramycin, as was demonstrated by inhibition of amino acid incorporation in a cell-free system by this drug. No tobramycin inactivation by adenylation or acetylation has been found. Similarly streptomycin is not adenylated or phosphorylated in strain 8803 (Shahrabadi 1974, personal communication). Thus the other antibiotics have no apparent mechanism of resistance.

Fifthly, there is no other detectable mechanism to account for the gentamicin resistance, as there is no evidence of gentamicin inactivation, nor of ribosomal resistance. In addition, despite multiple attempts, no evidence of transferable R-factor could be detected. Thus no other explanation of resistance is available.

Extrachromosomal DNA, isolated from strain 8803 and 13934 may not determine the diminished permeability, since strain 1136 which contain no extrachromosomal DNA gave the same pattern of gentamicin uptake. The function of these plasmids requires further study.

Uptake of gentamicin in the presence of secondary agents that modify the cellular envelope should be done to assess whether it is the cell wall or the cell membrane that forms the permeability barrier in these strains. For example, if there is an increase in gentamicin uptake after EDTA treatment, it could mean that the barrier of gentamicin-resistance is external to the peptidoglycan layer. Similarly, an increase of gentamicin uptake in the presence of carbenicillin would show that the peptidoglycan polymer acts as an important barrier to gentamicin penetration in these gentamicin-resistant strains. If no

difference exists between spheroplasts and whole cell gentamicin uptake it may be that the cell membrane forms the permeability barrier.

Our studies demonstrate that in a population of P. aeruginosa selected from hospitalized patients, all of the strains reported as resistant are so owing to defective permeability to gentamicin. This resistance presents a difficulty in the medical treatment, but it may be overcome by modification of the permeability barrier in the bacteria by a second antibiotic that can alter the barrier of permeability.

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